Regulation of 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase by Tocotrienols Isolated from Rice Bran Oil: Antiatherogenic Impacts in Normolipidemic and Hyperlipidemic Human and Animal Models

THESIS

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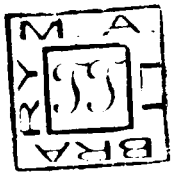

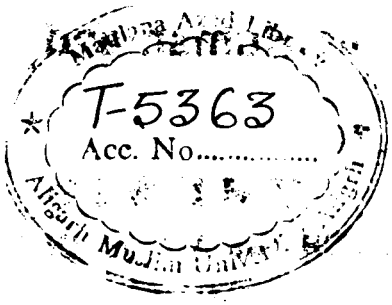

Dedicated to the memory of my father

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Certificate

This is to certify that the thesis entitled **'Regulation of 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase by Tocotrienols Isolated from Rice Bran Oil: Antiatherogenic Impacts in Normolipidemic and Hyperlipidemic Human and Animal Models'** herewith submitted by **Mohd. Minhajuddin, M.Sc.** in fulfilment of the requirements for the degree of **Doctor of Philosophy in Biochemistry** of the Aligarh Muslim University, is an authentic record of the research work carried out by him under my supervision and guidance and that no part, thereof, has been presented before for any other degree.

Aligarh
December, 1999

Z.H. Beg
Supervisor

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(Mohd. Minhajuddin)

CONTENTS

List of Abbreviations

Apo	Apoprotein
CAD	Coronary artery disease
CHD	Coronary heart disease
ELISA	Enzyme linked immunosorbant assay
FH	Familial hypercholesterolemia
HDL	High density lipoprotein
HLP	Hyperlipidemic
HMG-CoA	3-Hydroxy-3-methhylglutaryl coenzyme A
IDL	Intermediate density lipoprotein
LDL	Low density lipoprotein
NLP	Normolipidemic
RBO	Rice bran oil
T	Tocopherol
T_3	Tocotrienol
TAG	Triacylglycerol
TBARS	Thiobarbituric acid reactive substances
TRF	Tocotrienol rich fraction
VLDL	Very low density lipoprotein

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Introduction

Coronary artery disease (CAD) is one of the most important disease, in terms of both mortality and morbidity. According to a WHO and World Bank report nearly 2 million people in India die every year due to combined cardiovascular diseases. In addition, compared to Americans, Indians have 150 per cent to 400 per cent higher death rate from heart attacks. Population surveys carried out in the last few decades indicate that the prevalence of coronary artery disease (CAD) and hypertension has increased at least twofold in the last 20 years in both rural and urban populations in India (Dewan *et al.*, 1974; Gupta and Malhotra, 1975; Gupta *et al.*, 1995; Singh *et al.*, 1995b). In the urban population, the prevalence of hypertension and CAD is 3-4 times higher than in rural subjects. In South India (Malhotra, 1967; Raman *et al.*, 1993), the prevalence rates are higher in the urban as well as the rural population. CAD is also more common in males than females. It seems that differences in diet and lifestyle and the aging of populations may be important in the pathogenesis of hypertension and CAD in different population groups of Indians (Beegom and Singh, 1995; Singh *et al.*, 1995a). In the United States alone, CAD accounts for fully one-half of the nearly 1 million deaths each year from cardiovascular disease, and is the leading cause of death in both genders (American Heart Association, 1994). Each year, about 1.5 million Americans suffer acute myocardial infarction, and almost all myocardial infarctions are due to atherosclerosis of the coronary arteries. Among the two-thirds who survive the myocardial infarction, about two-thirds do not make a full recovery. In 19 per cent of Americans aged 15 years or older who are categorized as disabled, the disability is from CAD or other cardiovascular disease (American Heart Association, 1994).

Risk factor reduction is the primary clinical approach to preventing CAD morbidity and mortality. Epidemiological studies have clearly demonstrated that risk factors such as dyslipidemia, hypertension, and the use of tobacco products

act in a synergistic manner (Anderson *et al.*, 1991). High levels of total cholesterol, TAG, LDL-cholesterol, VLDL-cholesterol, apoB, Lp(a) and low levels of HDL-cholesterol, HDL_2-cholesterol, HDL_3-cholesterol, apoA-I are some of the lipid risk factors associated with CAD. Other non-lipid risk factors include physical inactivity, obesity, family history of CAD, age, gender, homeostatic factors, homocysteinemia, alcohol consumption, and psychological factors. The identification of risk factors provide a means for decreasing CAD risk, through the reduction of modifiable risk factors, and for informing treatment decisions, through more accurate determination of overall risk status. The understanding of risk factors and their relationship to the incidence of CAD evolved from prospective epidemiological studies in United States and Europe (Dawber *et al.*, 1957; Report of the Working Group on Arteriosclerosis of the NHLBI, 1981; Cooper, 1993; American Heart Association, 1994). These studies identify consistent associations between characteristics observed at one point of time in apparently healthy individuals, with the subsequent development of CAD in them. Probably the single most important contributory factor that has been implicated by various epidemiological studies in the development of CAD, is hyper-cholesterolemia.

Cholesterol which is widely distributed in the animal kingdom occurs in free form (unesterified) in all cell membranes (Myant, 1981), while in the plasma most of the cholesterol occurs in esterified form. Cholesterol has many biological functions. For instance, the concentration of cholesterol influences the fluidity of cell membranes and thereby biological activities of the cell. The cholesterol acts as a precursor for the synthesis of bile acids and steroid hormones. The total body cholesterol is derived from two sources: (i) dietary and (ii) *de novo* biosynthesis. Cholesterogenesis mostly occurs in the liver, which also regulates the level of circulating plasma cholesterol and serum lipoproteins. The biosynthesis of

cholesterol also occurs in the other organs like the intestines, adrenal cortex, reproductive organs and skin. Although other cells and tissues do not synthesize cholesterol, they have the genomic information for its synthesis. Under normal circumstances, these cells and tissues take up cholesterol from serum lipoproteins.

Starting from the 2-carbon unit acetyl-CoA, the biosynthesis of the cholesterol proceeds to several intermediates, including mevalonate and isopentenyl pyrophosphate (Fig. 1.1). Isopentenyl pyrophosphate is further processed in a series of steps to two branched pathways, one leading to isopentenyl tRNA and isopentenyl adenine, and the other to farnesyl pyrophosphate. Farnesyl pyrophosphate is in turn channeled to synthesis of cholesterol, ubiquinone or dolichols (Ross and Glomset, 1973; Brown and Goldstein, 1983). The observation that cancer cells lose feedback control of cholesterol biosynthesis, show elevated cholesterol levels, and exhibit a higher rate of cholesterogenesis provided the first indication of the link between cholesterol biosynthesis and cancer cell growth (Coleman and Laviets, 1981). The subsequent realization that not only cancer cells but also preneoplastic and normal proliferating cells show elevated levels of cholesterol as well as higher rates of cholesterogenesis indicated that cholesterol biosynthesis is likely to play an important role not only in carcinogenesis but also normal cell growth (Rao, 1986).

Hepatic level of cholesterol are maintained by a precised balance between reactions catalyzed by 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase, EC 1.1.1.34), cholesterol 7-α hydroxylase, acyl CoA : cholesterol acyltransferase and cholesteryl ester hydrolase. The first two enzymes are the rate limiting enzymes for cholesterol and bile acid synthesis, respectively. Acyl CoA : cholesterol acyltransferase catalyzes esterification of free cholesterol, whereas cholesteryl ester hydrolase mediates release of free cholesterol from stored cholesteryl esters.

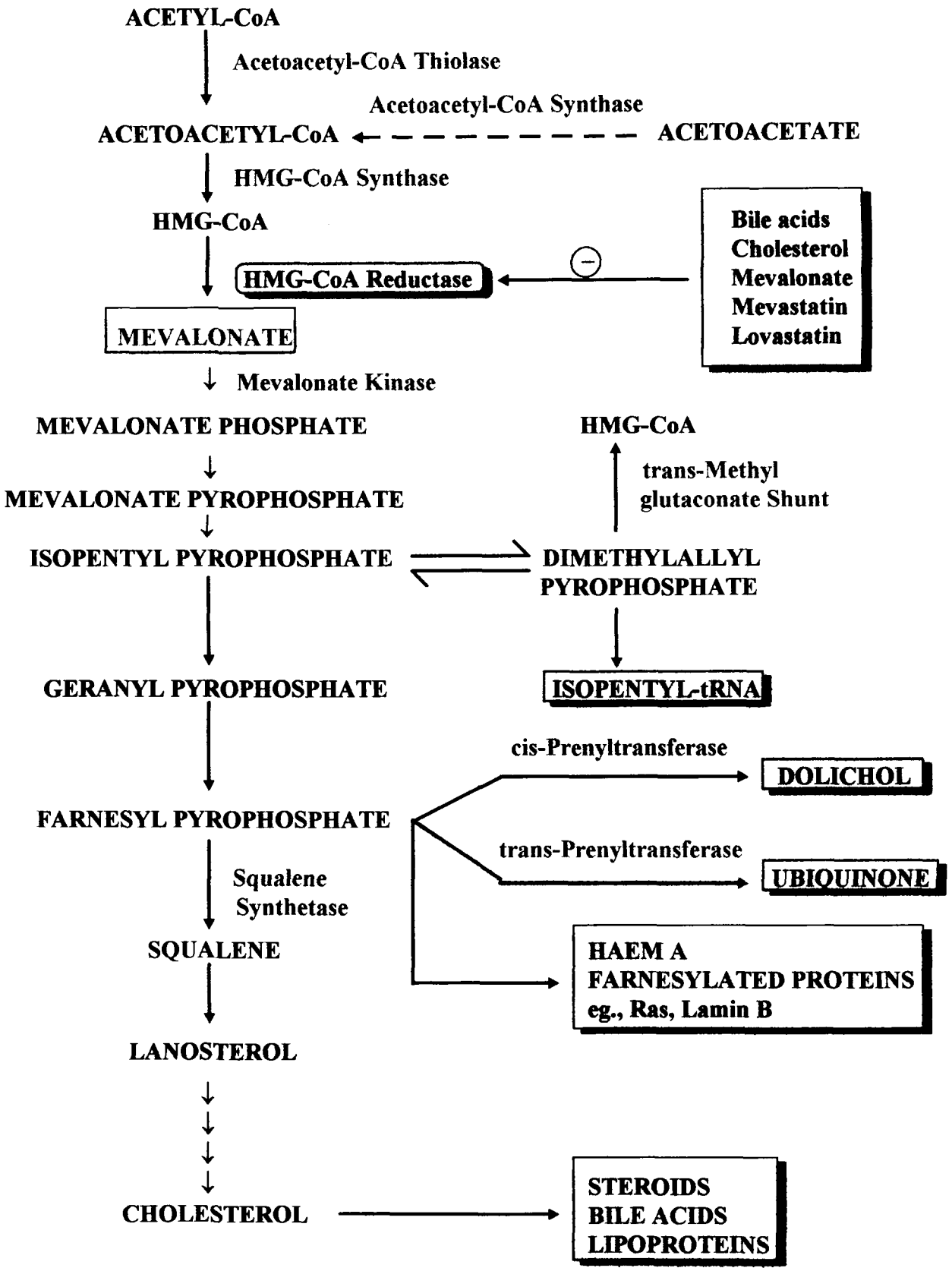


Fig 1.1. The biosynthetic pathways of mevalonate, sterols and isoprenoid compounds.

The primary feed-back loop for regulation of cholesterol synthesis appears to be at the site where HMG-CoA is converted to mevalonic acid by the rate limiting enzyme HMG-CoA reductase (Luskey, 1991). Cholesterol and other oxysterols inhibit the activity of HMG-CoA reductase. Because of the strong link between cholesterol and coronary heart disease and its link with cancer (Rao, 1986), there is currently a renewed interest in studying the regulation of HMG-CoA reductase and several other key enzymes in cholesterol biosynthetic pathway. HMG-CoA reductase regulates the synthesis of cholesterol and other polyisoprenoid compounds (Rodwell *et al.*,1976; Goldstein and Brown, 1979; Brown and Goldstein, 1979; Brown and Goldstein, 1980; Beg *et al.*, 1981; Beg and Brewer, 1982). In mammalian cells, HMG-CoA reductase is a transmembrane glycoprotein with its active site facing the cytosol and a carbohydrate containing site oriented toward the luminal surface of the endoplasmic reticulum (Liscum *et al.*, 1983a; Brown and Simoni, 1984). HMG-CoA reductase is an approximately 100 kDa protein (Chin *et al.*, 1982; Edwards *et al.*, 1983a; Hardeman *et al.*, 1983; Chin *et al.*, 1984; Beg *et al.*, 1985). Proteolysis of the native protein results in a 53 kDa molecular weight fragment that contains the active site of the enzyme (Chin *et al.*, 1982; Liscum *et al.*, 1983a; Edwards *et al.*, 1983a; Hardeman *et al.*, 1983; Chin *et al.*, 1984; Beg *et al.*, 1985). HMG-CoA reductase is a protein of 887 amino acids containing three potential sites for aspargine-linked glycosylation. The N-terminal half of the peptide is anchored to the membrane and contains seven hydrophobic regions, each of which is comprised of 20 amino acids and spans the microsomal membrane (Chin *et al.*, 1984). The N-terminal lacks the signal sequence and the hydrophilic C-terminal half of HMG-CoA reductase contains the catalytic site of the enzyme (Chin *et al.*, 1984). Since, the catalytically active hydrophilic tail of the enzyme extends into the cytoplasm, it is more accessible to the action of modulators and permits the observed multifaceted regulation of

HMG-CoA reductase and cholesterol synthesis. The complex homeostatic mechanism by which the enzyme activity of HMG-CoA reductase and cholesterol biosynthesis are coordinately regulated in response to various physiological stimuli has been extensively studied.

Isolation, purification, and characterization of rat hepatic HMG-CoA reductase has been well documented (Kawachy and Rudney, 1970; Heller and Gould, 1973; Brown *et al.*, 1973; Heller and Gould, 1974; Heller and Shrewsburg, 1976; Kleinsek *et al.*, 1977; Srikantaiah *et al.*, 1977; Edwards *et al.*, 1979). HMG-CoA reductase has also been studied in several other species including chicken liver (Beg *et al.*, 1978; Beg *et al.*, 1979), human liver (Beg *et al.*, 1982a; Beg *et al.*, 1982b) and human fibroblasts (Brown and Goldstein, 1983; Beg *et al.*, 1987). Several different mechanisms for the regulation of enzymes in metabolic pathways have been elucidated such as modulation by isosteric and allosteric effectors, regulation of enzyme synthesis and degradation, feed-back control, and covalent modification (Siperstein, 1970; Holzer and Duntze, 1971; Segal, 1973; Carlson and Kim, 1973; Lee *et al.*, 1976; Greengard, 1978). Three basic control mechanisms for HMG-CoA reductase have been reported. (a) Long-term regulation, which involves the modulation of HMG-CoA reductase activity by changes in enzyme concentration through the regulation at transcriptional level and post-transcriptional regulatory mechanisms such as mRNA and enzyme protein degradation (Kirsten and Watson, 1974; Jakoi and Quarfordt, 1974; Chan *et al.*, 1981; Koizumi *et al.*, 1982; Faust *et al.*, 1982; Edwards *et al.*, 1983a; Edwards *et al.*, 1983b; Liscum *et al.*, 1983b; Clarke *et al.*, 1983; Sinensky and Logel, 1983; Clarke *et al.*, 1984). For instance, the product feedback regulation by mavalonate (Kita *et al.*, 1980; Brown and Goldstein, 1980; Cohen *et al.*, 1982), *in vivo* inhibition of HMG-CoA reductase by cholesterol feeding (Arebalo *et al.*, 1981), cholestyramine and mevinolin (Eisenberg and Levy, 1975), and mevalonolactone

(Arebalo *et al.*, 1980; Beg *et al.*, 1984) has been reported. Tocotrienols, a naturally occurring class of compounds of vitamin E family, have also been reported to regulate HMG-CoA reductase activity at the post-transcriptional level (Pearce *et al.*, 1992; Parker *et al.*, 1993). (b) Control of HMG-CoA reductase activity through changes in the membrane composition and membrane fluidity in the microsomal environment in the immediate vicinity of the enzyme (Finkel and Volpe, 1979; Mitropoulous *et al.*, 1981; Siptal and Sabine, 1981; Richert *et al.*, 1984). (c) Short-term regulation, that involves reversible covalent modification (phosphorylation and dephosphorylation) of HMG-CoA reductase (Ingebritson and Gibson, 1980; Beg *et al.*, 1981; Beg and Brewer, 1982; Kennelly and Rodwell, 1985). Three separate kinase systems for the regulation of HMG-CoA reductase involving short-term covalent modification have been demonstrated (Ingebritson and Gibson; 1980, Beg *et al.*, 1987). Studies involving incubation of rat hepatocytes with insulin and glucagon or administration of glucagon to rats have been shown to modulate the bicyclic cascade system involving phosphorylation of both HMG-CoA reductase and HMG-CoA reductase kinase (Ingebritson and Gibson, 1980; Gibson, 1985; Beg *et al.*, 1987; Gibson and Parker, 1987).

Although cholesterol is essential to life, excess or deficit of free cholesterol is known to be harmful. Several factors are known which cause an overall increase in cholesterol concentration in the liver (Fig. 1.2) (i) uptake of lipoproteins by receptor mediated endocytosis, (ii) non-receptor mediated intake of lipoproteins, (iii) uptake of free cholesterol from the cholesterol rich lipoproteins by cell membranes, (iv) *de novo* synthesis of cholesterol, and (v) hydrolysis of cholesterol esters by cholesteryl ester hydrolase. Under above situations, not only increased cholesterol levels inhibits its own synthesis by inhibiting HMG-CoA reductase and suppressing low-density lipoprotein (LDL) receptors (Russel, 1983), but also by activating cholesterol 7-α hydroxylase and acyl CoA : cholesterol acyltransferase

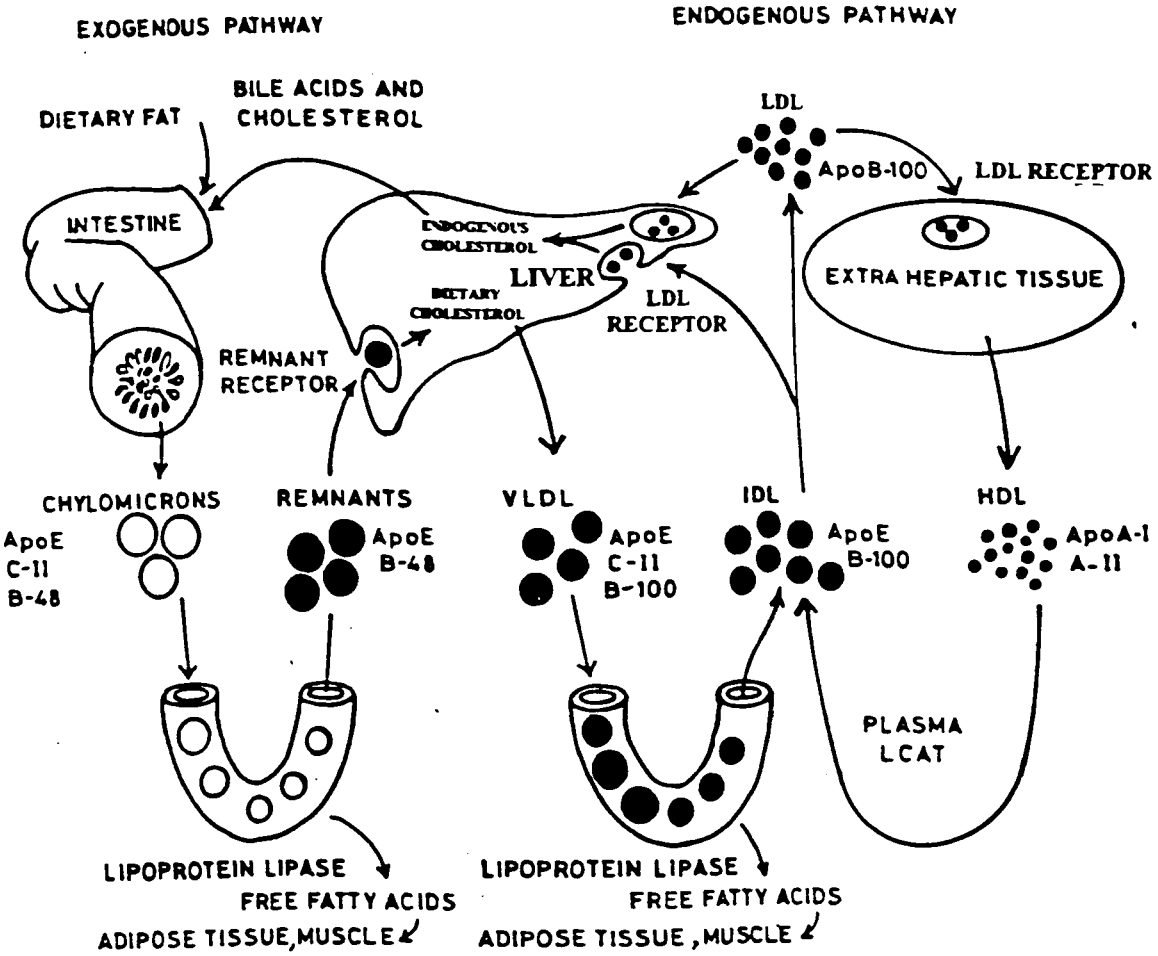


Fig. 1.2. A model for plasma triacylglycerol and cholesterol transport.
(Adapted from Biochemistry by D. Voet and J.G. Voet, J. Willey and sons, New York, 1990 ed., p-306).

which utilize free cholesterol for bile acid synthesis and formation of cholesteryl esters, respectively. Conversely, some factors are involved in the decrease of hepatic cholesterol, they are (a) efflux of cholesterol from membrane to nascent high-density lipoproteins (HDL) and HDL_3 which is catalyzed by lecithin : cholesterol acyltransferase, (b) esterification by acyl CoA : cholesterol acyltransferase and (c) utilization of cholesterol for synthesis of steroids and bile acids. Under these conditions increase in cholesterol is achieved by activation of HMG-CoA reductase and cholesteryl ester hydrolase activities as well as induction in synthesis of LDL receptors in order to receive cholesterol from non-hepatic tissues by receptor mediated endocytosis (Fig. 1.2). However, under normal conditions an intricate balance is maintained between the biosynthesis, utilization and transport of cholesterol, keeping its harmful effects to minimum.

Lipids are transported through plasma compartment in lipoproteins, which are complex water soluble molecules consisting of a core of cholesteryl esters and triacylglycerols (TAG) covered by a surface monolayer of phospholipids, free cholesterol and apolipoproteins. In the last two decades, there have been major advances in our understanding of the role of plasma lipoproteins, apolipoproteins, lipolytic enzymes, and lipoprotein receptors in cholesterol and lipoprotein metabolism. This new information has provided major insights into the role of cholesterol and lipoproteins in the pathogenesis of premature atherosclerosis. There are six major classes of human plasma lipoproteins, these include chylomicrons, very low density lipoproteins (VLDL), intermediate-density lipoprotein (IDL), low-density lipoprotein (LDL), high density lipoprotein (HDL) and lipoprotein (a) [Lp(a)] (Gofman *et al.*, 1954; Berg *et al.*, 1974). HDL can be further separated by hydrated density into HDL_2 and HDL_3. These lipoproteins are distinguished on the basis of their lipid content, ultra-centrifugation size, electrophoretic mobility and surface proteins.

Fourteen major human plasma apolipoproteins have been identified and their gene and protein structures determined (Brewer *et al.*, 1988). The five most clinically relevant apolipoproteins are A-I, B-100, B-48, C-II and E. The two major apolipoproteins on HDL are apoAI and apoAII (Oran *et al.*, 1983; Suzuki *et al.*, 1983). In human plasma apoB exist as two isoproteins designated apoB100 and apoB-48, with molecular weights of 512 and 250 kDa, respectively (Kane *et al.*, 1980; Kane, 1983). ApoB-48 and apoB-100 are the principal structural apolipoproteins on chylomicrons, VLDL, IDL and LDL. ApoB-100 is virtually the only apolipoprotein on LDL.

Over the last decade three major physiological functions for the plasma apolipoproteins have been identified.

(i) Apolipoproteins functions as structural proteins for the biosynthesis and secretion of plasma lipoproteins. ApoB-100 and apoB-48 are required for the secretion of TAG-rich lipoproteins from the liver and intestine. ApoA-I has been proposed to be an important structural protein for the biosynthesis of HDL. Individuals with an inability to synthesize and secrete apoA-I have a virtual absence of plasma HDL (Narun *et al.*, 1982; Schaefer *et al.*, 1985).

(ii) Apolipoproteins function as cofactors or activators of enzymes involved in lipid and lipoprotein metabolism. ApoC-II is required for the enzymic activity of lipoprotein lipase, which is responsible for the perivascular hydrolysis of lipoprotein TAG to free fatty acids and monoacylglycerols (La Rosa *et al.*, 1970; Havel *et al.*, 1970). Lipoprotein lipase is attached to the capillary endothelium by a heparin-like proteoglycan allowing direct interaction of the enzyme with the circulating TAG-rich lipoproteins. A deficiency of lipoprotein lipase or apoC-II results in defective TAG hydrolysis (Breckennidge *et al.*, 1978). Clinically, patients have eruptive xanthomas, severe hypertriglyceridemia and recurrent bouts of pancreatitis. ApoA-I activates lecithin : cholesterol acyltransferase, which

catalyzes the esterification of plasma cholesterol to cholesteryl esters (Fielding *et al.*, 1972).

(iii) Apolipoproteins also play a critical role in lipoprotein metabolism as ligands on lipoprotein particles which interact with cellular receptors for specific lipoproteins. ApoB-100 interacts with the LDL receptor to initiate absorptive endocytosis and cellular uptake of LDL (Brown and Goldstein, 1986). ApoE has been proposed to interact with apoE receptor which facilitates the hepatic removal of lipoprotein remnants secreted by the intestine and liver (Davignan *et al.*, 1988; Herz *et al.*, 1988).

The metabolic relationship of the major classes of lipoproteins containing apoB-48 and apoB-100 may be considered to consist of two major "apoB cascades". The first apoB cascade involves the stepwise delipidation of TAG-rich chylomicrons secreted by the intestine. These lipoproteins transport dietary cholesterol and TAG from the intestine to the liver and peripheral tissues. Shortly after secretion, chylomicrons acquire apolipoproteins C-II and E primarily from HDL. As already outlined, apoC-II activates lipoprotein lipase, which initiates TAG hydrolysis and remodeling of the lipoprotein particles. With TAG hydrolysis of the hydrated density the chylomicrons increases and chylomicron remnants are generated with a hydrated density of VLDL and then IDL. Chylomicron remnants are removed primarily by a hepatic remnant receptor (Davignan *et al.*, 1988) ApoE has been proposed to interact and initiate the hepatic uptake of the chylomicron remnants (Davignan *et al.*, 1988; Hertz *et al.*, 1988). The second apoB cascade is a parallel cascade involving TAG-rich VLDL containing apoB-100 secreted by the liver. ApoC-II and apoE from HDL rapidly associate with the newly secreted hepatogenous VLDL. ApoC-II activates lipoprotein lipase, which hydrolyzes VLDL triglycerides, and the VLDL are serially converted to smaller VLDL remnants, IDL, and finally LDL. During the conversion of VLDL to LDL

approximately 50 per cent of VLDL remnants and IDL are removed directly from the plasma by interaction of apoE and apoB-100 with the remnant and LDL receptors.

Another lipolytic enzyme, hepatic lipase and apoE have also been proposed to be necessary for the conversion of IDL to LDL. Hepatic lipase functions as both a triglyceryl hydrolase and phospholipase. LDL, the end product of the VLDL cascade, contains almost exclusively apoB-100 as its only protein constituent. ApoB-100 on LDL interacts with LDL receptor on the plasma membrane of cells in the liver, adrenal and peripheral cells, including smooth muscle cells and fibroblasts (Brown and Goldstein, 1986), where it supplies cholesterol to the intracellular cholesterol pool. The work of Brown and Goldstein (1979 & 1983) on the cellular metabolism of LDL elucidated the *LDL Pathway*. The high affinity receptors bind LDL particles and extract them from the fluid that bathes the cell. LDL is transported to lysosomes where the protein is degraded and the cholesterol is transferred to the intracellular cholesterol pool (Brown and Goldstein, 1986). The receptors displayed on the surface of the cells varies with the cellular demand for cholesterol. When the need is low, the cells make fewer receptors and take up LDL at a reduced rate, this protects the cells excess cholesterol but at a higher price, the reduction in the number of receptors decreases the rate of removal of LDL from the circulation, blood level of LDL rises and atherogenesis is accelerated. LDL receptor play an important role in the maintenance of plasma LDL-cholesterol levels. The serum concentration of LDL, therefore, depends on the rate that liver removes IDL from the circulation which inturn, depends on the number of functioning LDL receptors on the liver cell surface (Fig. 1.3a). High blood cholesterol which results from the overproduction and/or underutilization of LDL, is known to be caused by two metabolic irregularities (i) the genetic disease familial hypercholesterolemia (discussed in detail later); (ii) the consumption of

high cholesterol diet. Familial hypercholesterolemia (FH) (Fig. 1.3b) is a dominant genetic defect that results in a deficiency of functional LDL receptors. FH homozygotes, therefore have plasma LDL-cholesterol levels three to five times higher than average. FH heterozygotes, which are far more common, have about one half of the normal number of functional receptors and plasma LDL-cholesterol levels of about twice the average. The ingestion of a high cholesterol has an effect similar, although not as extreme, as FH (Fig. 1.3c). Excessive dietary cholesterol enters the liver cells in chylomicron remnants and repress the synthesis of LDL-receptor protein. The resulting insufficiency of LDL-receptors on the liver cell surface has consequenses similar to those of FH. In WHHL rabbits, with a genetic deficiency of LDL receptor function, extremely high plasma LDL cholesterol levels are observed with the development of atherosclerosis early in life (Brown and Goldstein, 1983). LDL receptor activity is under the metabolic regulation *in vivo*, such that receptor activity can be increased or decreased by appropriate interventions with diet and/or drugs (Mahley and Innerarity, 1983; Brown and Goldstein, 1983).

Nascent HDL, primarily in the form of phospholipid-apolipoprotein A-I discs, are synthesized both in the human liver and intestine. The nascent HDL acquire cholesterol from tissues and the enzyme lecithin : cholesterol acyltransferase catalyzes the esterification of cholesterol to cholesteryl esters. With the increase in lipid content, the nascent HDL are converted to HDL_3, these HDL_3 lipoproteins are then converted to the larger HDL_2 lipoproteins by the acquisition of lipids and apolipoproteins released during the stepwise delipidation and remodeling of the TAG rich chylomicrons and VLDL as well as the uptake of cholesterol from peripheral tissues. HDL_2 is converted back to HDL_3 by the removal of TAG and phospholipids by hepatic lipase as well as by the transfer of cholesteryl esters into VLDL and LDL by the cholesteryl ester exchange protein

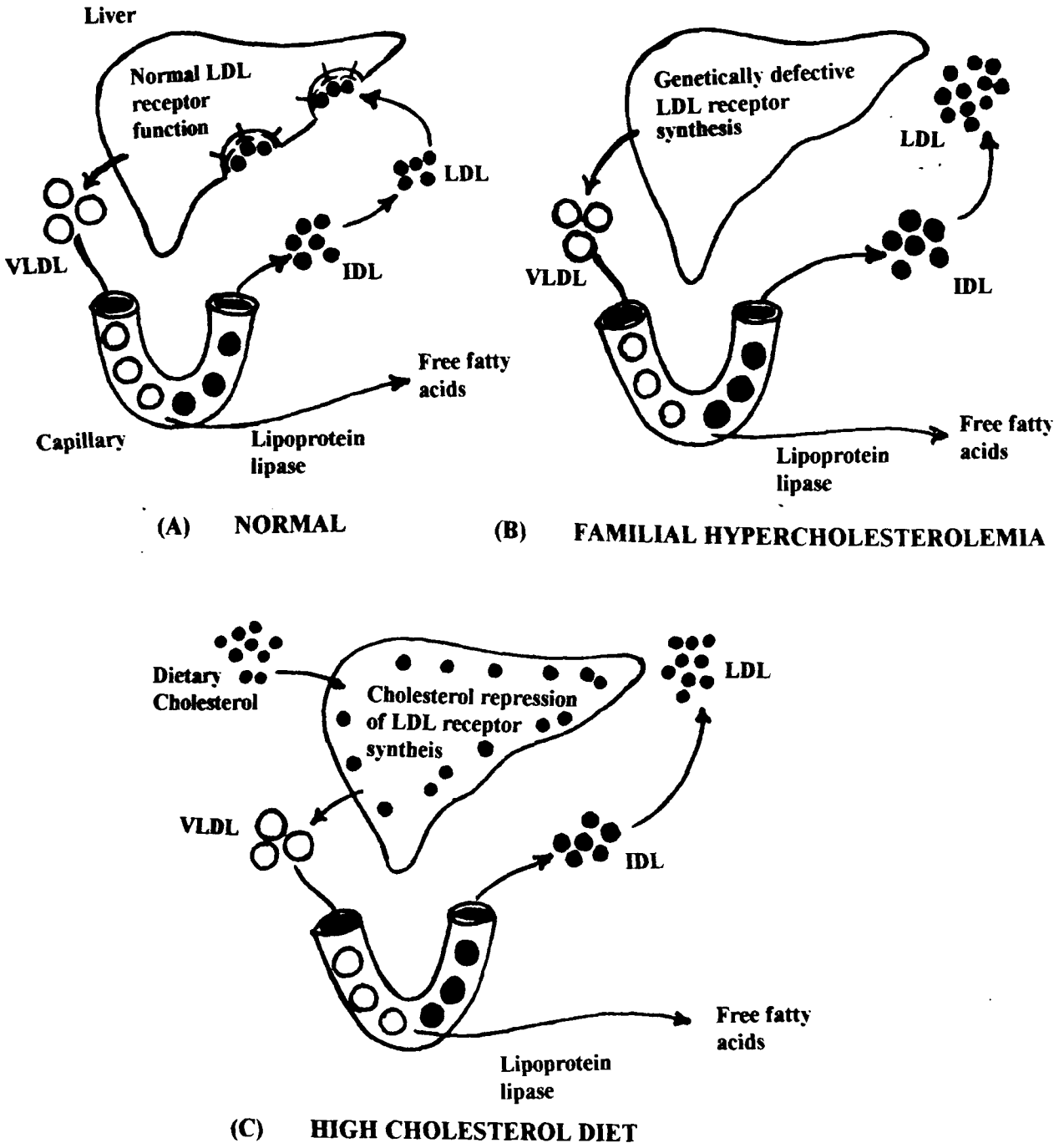


Fig. 1.3. Liver LDL receptors control plasma LDL production and uptake. (A), Normal human subjects; (B), Individuals with Familial hypercholesterolemia and (C), Individuals who ingest a high cholesterol diet.
(Adapted from Biochemistry by D. Voet and J.G. Voet, J. Willey and sons, New York, 1990 ed., p-306).

(or lipid transfer protein) as well as the transfer of cholesteryl esters to the liver and other tissues. In this overall process, HDL are interconverted from HDL_3 to HDL_2 and back to HDL_3 as cholesterol is picked up and transferred from peripheral tissues to the liver (Fig. 1.4). This process is termed as *reverse cholesterol transport* (Brewer *et al.*, 1971; Eisenberg, 1984). In this proposed model, HDL interacts with a putative HDL receptor (Oran *et al.*, 1983; Suzuki *et al.*, 1983; Schmitz *et al.*, 1988) that facilitates the transfer of intracellular cholesterol to HDL. HDL transports this cholesterol in plasma and delivers it to the liver via the HDL receptor for removal from the body by direct secretion into bile or following conversion to bile acids. A variable portion of tissue cholesterol has been proposed to be transported to the liver by HDL particles containing apoE, which may interact with the hepatic remnant and LDL receptors (Eisenberg, 1984).

Atherosclerosis, which is the most common form of arteriosclerosis (hardening of arteries), is characterized by the presence of atheromas. These atheromas or arterial thickenings exude a paste of yellow deposit of almost pure cholesteryl esters upon sectioning. Atherosclerosis is a progressive disease and results due to the deposition of intracellular lipids in the smooth muscle cells of the inner arterial wall. These lesions narrow and eventually block the arteries due to the formation of fibrous, calcified plaques. The rough arterial wall promotes the formation of blood clots, which may also block the artery. Due to the blocking of arteries, blood flow stops and causes the death of the deprived tissues. The stoppage of blood flow is known as an infarction. Atheromas mostly occur in the arteries supplying blood to the heart known as coronary arteries. This results in myocardial infarctions or heart attacks, which is the most common cause of death in Western man (Packer and Landvik, 1989). The earliest lesions of atherosclerosis can be found in young children and infants in the form of a lesion called the *fatty streak*, whereas the advanced lesion, the *fibrous plaque*, generally appears during early adulthood

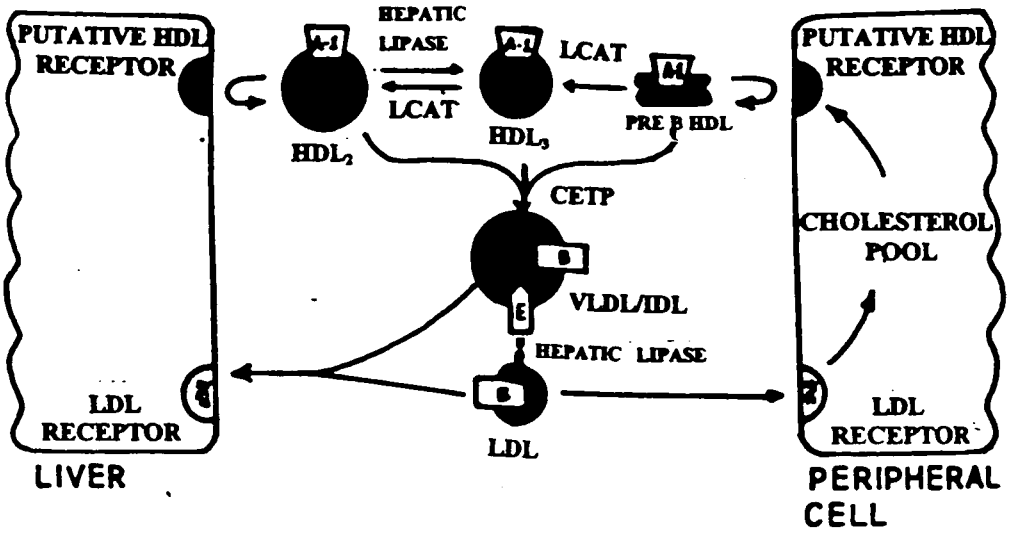


Fig. 1.4. General overview of "reverse cholesterol transport".
(Adapted from Atherosclerosis and Coronary Artery Disease, edited by V. Fuster, R. Ross and E.J. Topol, Lippincott-Raven Publishers, Philadelphia, 1996, p-71).

and progresses with age (Geer *et al.*, 1961; Geer, 1965; Ghidoni and Oniel, 1967; Stary, 1983; Stary *et al.*, 1994; American Heart Association, 1994). Fatty streaks were first observed by Stary (1983), he demonstrated that by the age of ten years, the fatty streaks consisted principally of lipid laden macrophages and smooth muscle cells and appears as yellow coloured area due to large amount of lipid filled foam cells. The bulk of lipid is generally formed by cholesteryl esters, cholesterol derived from lipoproteins.

Stary (1983) also studied fatty streaks in coronary arteries of children and young adults and observed that they were localized at same anatomical sites as the fibrous plaques did in older individuals. Based on this observation he concluded that the fatty streak with age advanced to form the mature fibrous plaque in adults. Chemical analysis of severe atherosclerotic plaques from humans and animal models indicate that on an average, fully developed atheromas are composed of about one half lipid and one half protein, including the cellular and extra cellular proteins, however, there may be great variations in these elements.

Two decades ago, atherosclerosis was considered to be a degenerative process because of the accumulation of lipid and necrotic debris in the advanced lesions, it is, however, now recognized that it is infact a multifactorial phenomena. The form and content of the advanced lesions of atherosclerosis demonstrates results of three fundamental biological processes; accumulation of intimal smooth muscle cells, together with variable numbers of macrophages and T-lymphocytes, formation of large amount of connective tissue matrix, including collagen, elastic fibers, and proteoglycans by the proliferated smooth muscle cells and accumulation of lipids, principally in the form of cholesteryl ester and free cholesterol within the cells as well as in the surrounding tissues. (Ross and Glomset, 1973; Ross and Glomset, 1976; Fuster *et al.*, 1992; Ross and Fuster, 1996).

Jackson and Gotto (1976) have suggested that injury to endothelial cell membrane at arterial wall, may occur upon exposure to chronically elevated levels of LDL due to an increase in the number of cholesterol molecules in plasma membranes of cells, including endothelial cells. Elevation of the cholesterol/ phospholipid ratio of endothelial cell plasma membrane can theoretically lead to an increase in the viscosity and decrease in the malleability of endothelial cell surface, which has a critical effect at particular anatomical sites, such as points of bifurcation in the arterial tree. This could lead to inter-endothelial cell separation and retraction particularly at the sites where the blood flow is modified due to the formation of fatty streaks, as has been found in hypercholesterolemic monkeys, swine and humans.

Repeatedly epidemiological studies have established a strong association between elevated blood cholesterol and CAD. Risk for CAD is increased with increasing plasma cholesterol levels and can be decreased by decreasing plasma cholesterol. One of the important observational study demonstrating a positive relation between total cholesterol level and CAD mortality was "The Multiple Risk Factor Intervention Trial" (Stamler *et al.*, 1986). It was observed that in Japan and some other Mediterranean countries, where the dietary intake of saturated fat and mean plasma cholesterol level was relatively low, the mortality rate for CAD was also low. As against this, countries like Finland and United States, with a high average plasma cholesterol levels showed a higher CAD mortality rate (Keys, 1970). An important interventional study on primary prevention of CAD was the Lipid Research Clinic Coronary Primary Prevention Trial. The hypercholesterolemic subjects of this study received either bile acid sequestrant, cholestyramine or a placebo (LRCP, 1984a). The cholestyramine group, showed significant reductions in LDL-cholesterol. Incidence of non-fatal myocardial infarction and CAD death was also reduced significantly (LRCP, 1984b) in this group. The result

of Lipid Research Clinic Coronary Primary Prevention Trial provided the first major clinical substantiation to the lipid hypothesis, and were also the first to give rise to the rule of thumb that a 1% decrease in total cholesterol reduces the incidence of CAD events by 2 to 3%.

Increased plasma levels of three lipoproteins, LDL (Gorton *et al.*, 1977; Castelli *et al.*, 1977), β-VLDL (Mehley, 1979; Brewer *et al.*, 1983) and Lp(a) (Berg *et al.*, 1974; Kostner *et al.*, 1981; Armstrong *et al.*, 1986) and decreased levels of HDL (Castelli *et al.*, 1977; Miller *et al.*, 1977; Naito, 1980; Yaari *et al.*, 1981) have been associated with the development of premature cardiovascular disease. Increased plasma levels of LDL may result from ingestion of excess dietary saturated fat and cholesterol as well as from primary and secondary hyperlipoproteinemias. β-VLDL are abnormal lipoprotein remnant particles present in patients with type-III hyperlipoproteinemia and animals fed a high cholesterol and fat diet (Mahley, 1979; Havel, 1982; Brewer *et al.*, 1983). β-VLDL remnant lipoproteins have been proposed to be taken up by macrophages by either the LDL receptor (Koo *et al.*, 1988) or a specific macrophage β-VLDL receptor (Venlenter *et al.*, 1983; Baker *et al.*, 1984). Elevated levels of Lp(a) are genetically determined and associated with an increased risk of premature cardiovascular disease (Berg *et al.*, 1974; Kostner *et al.*, 1981; Armstrong *et al.*, 1986). The mechanism for the potential cellular uptake of Lp(a) and the development of premature cardiovascular disease is not known. Over the past 30 years a considerable body of evidence has accumulated to establish that an elevated concentration of the Lp(a) lipoprotein, commonly referred to as "lipoprotein little a" in plasma is an important independent risk factor for the development of premature cardiovascular disease (Berg *et al.*, 1974; Kostner; 1981; Armstrong *et al.*, 1986). Lp(a) levels in plasma range from less than 1 to more than 100 mg/dl. Approximately 20 per cent of the population have levels

above 30 mg/dl; this is associated with a twofold increase in the relative risk of coronary atherosclerosis. With elevations of both Lp(a) and LDL, the relative risk of vascular disease increases to approximately fivefold (Armstrong *et al.*, 1986).

An inverse association has been established between HDL-cholesterol level and CAD incidence in numerous epidemiological studies. For example, in the Framingham Heart Study, men and women with HDL-cholesterol of 35 mg/dl or less had an eight fold increase in CAD incidence compared with men and women with HDL-cholesterol of 65 mg/dl or greater (Gordon *et al.*, 1977). Women have significantly higher plasma levels of HDL_2 than men (James and Pometta, 1990); increased levels of these larger, less dense particles may be partially responsible for the relative cardioprotection seen in premenopausal women. Each 1 mg/dl increase in HDL-cholesterol is estimated to decrease CAD risk 2 per cent in men and 3 per cent in women (Gordon *et al.*, 1989). The low HDL in men with clinically significant CAD is due to a reduction in both HDL_2 and HDL_3; however, the reduction in HDL_2 is greater than that in HDL_3 (Miller *et al.*, 1981b; Laakso *et al.*, 1985; Wallentin and Sundin, 1985; Hamsten *et al.*, 1986). These studies support the concept that HDL_2 is a better predictor of coronary artery disease than is HDL_3 (Drexel *et al.*, 1992). The question, however, as to whether quantitation of HDL_2 as a screening test is superior to total HDL-cholesterol has not been definitively answered due to variability in results of the individual reported studies.

The results of the studies in which apoA-I was evaluated as a discriminator for CAD also do not permit a definitive conclusion to be drawn as to whether apoA-I is a better discriminator than HDL-cholesterol. In the majority of patients with angiographically documented CAD or survivors of myocardial infarction, plasma apoA-I concentrations were lower than control groups (Brunzell *et al.*, 1984; Miller, 1987; Alaupovic *et al.*, 1988). In some studies apoA-I has been

shown to be a better discriminator than lipids and lipoprotein cholesterol levels in identifying patients with CAD, while in other studies the apoB/apoA-I ratio appeared to be an even more powerful predictor than the individual lipoproteins (Brunzell *et al.*, 1984; Miller, 1987; Alauporic *et al.*, 1988). However, in other studies HDL-cholesterol was found to be a better predictor than apoA-I (Brunzell *et al.*, 1984; Miller, 1987; Alauporic *et al.*, 1988).

The relation between plasma TAG and CAD is not as well established as the relation between plasma cholesterol and CAD. Epidemiological evidence, however, suggests that TAG plays an important role in determining CAD risk. In Helsinky Heart Study (Manninen *et al.*, 1992) it was established that the relative risk for cardiac events was significantly more in the subgroup with a higher TAG and LDL cholesterol : HDL cholesterol ratio as compared to the subgroup with lower TAG and LDL cholesterol : HDL cholesterol ratio. Diabetes is frequently associated with hypertriglyceridemia, and is often combined with reduced HDL-cholesterol (Haward, 1987). Recently, there has been increasing evidence of an association between TAG and increased risk of cardiovascular disease (Austin, 1991), a risk that is especially high in subjects with low HDL-cholesterol (Castelli, 1986). One of the major causes of hypertriglyceridemia with low HDL-cholesterol in diabetes is the decrease in lipoprotein lipase activity, an enzyme that plays crucial role in both TAG removal and HDL-cholesterol production, due to insulin deficiency, because lipoprotein lipase is known to be an insulin-dependent enzyme (Garfinkel *et al.*, 1976; Murase *et al.*, 1981).

A classification of dyslipidemias as recommended by Fredrickson is shown in Table A.

Table A

Phenotype	Lipoprotein(s) Elevated	Plasma Cholesterol Level	Plasma Triglyceride Level	Atherogenecity
I	Chylomicrons	Normal to ↑	↑↑↑↑	Not seen
IIa	LDL	↑↑	Normal	+ + +
IIb	LDL and VLDL	↑↑	↑↑	+ + +
III	IDL	↑↑	↑↑↑	+ + +
IV	VLDL	Normal to ↑	↑↑	+
V	VLDL and chylomicrons	↑ to ↑↑	↑↑↑↑	+

(Source: International Lipid Information Bureau: The ILIB Lipid Handbook for Clinical Practice: Blood Lipids and Coronary Heart Disease. Houston, International Lipid Information Bureau, 1995).

Elevated levels of plasma apoB and LDL cholesterol may be due to dietary excess, genetic hypercholesterolemias or other diseases. Approximately 10 per cent of the patients with hypercholesterolemia have a monogenic disease causing elevated plasma LDL levels. The monogenic disease, *familial hypercholesterolemia*, is an autosomal codominant disease characterized clinically by tendon and tuberous xanthomas, arcus, xanthelasma and premature cardiovascular disease (Brown and Goldstein, 1986). The molecular defect in familial hypercholesterolemia is one of several mutations in the gene coding for the LDL receptor (Brown and Goldstein, 1986). The deficiency of the LDL receptor leads to a decreased rate of removal of plasma LDL. The reduction in the number of LDL receptors has been previously demonstrated to be proportional to the elevated levels of plasma LDL (Sprecher *et al.*, 1985). The frequency of familial hypercholesterolemia is approximately 1 in 500 and the heterozygotes and homozygotes have plasma LDL cholesterol levels >250 mg/dl and >750 mg/dl, respectively. Plasma apoB levels are usually greater

than 140 mg/dl. The clinically important complication of familial hypercholesterolemia is premature atherosclerosis. In homozygous patients coronary and myocardial infarctions are frequent in young adults. For heterozygous men, the chance of having a myocardial infarction is approximately 5 per cent by age of 30, 51 per cent by the age of 50 and 85 per cent by 60.

Familial combined hyperlipidemia is probably the most frequent monogenic dyslipoproteinemia (frequency 1 in 300) in the population associated with an increased risk of premature cardiovascular disease (Goldstein *et al.*, 1973; Rose *et al.*, 1973). Clinically the patients may have arcus and xanthelasmas; however, tendon xanthomas are rare and this may be used as a diagnostic clue to distinguish these patients from patients with familial hypercholesterolemia. Plasma cholesterol and TAG levels are generally moderately elevated. A characteristic feature of combined hyperlipidemia is that the lipoprotein elevation is variable and may change from elevated LDL (phenotype IIa) to VLDL and LDL (phenotype IIb) and VLDL (phenotype IV). In addition, several different phenotypes may be observed in a single family. In contrast to familial hypercholesterolemia, the cholesterol levels may not be elevated in childhood and the hyperlipidemia frequently is not expressed until after age 30.

The molecular defect(s) in combined hyperlipidemia has not been elucidated. In the patients studied, the characteristic metabolic defect is the over production of apoB-containing lipoproteins (Tenz *et al.*, 1986). The LDL present in patients with combined hyperlipidemia is abnormal in hydrated density and lipid composition. The "dense LDL" characteristic of combined hyperlipidemia has a hydrated density of approximately 1.055 gm/ml and has a ratio of LDL cholesterol to LDL apoB of <1.3 as compared with a normal ratio of >1.3 (Sniderman *et al.*, 1980; Krauss *et al.*, 1988; Musliner and Krauss, 1988). The LDL apoB level in these patients is usually >120 mg/dl. The presence of "dense

LDL" may be an important causative factor in the development of atherosclerosis observed in these patients. Elevated plasma level occur in type III hyperlipoproteinemia and in experimental animals fed diets high in cholesterol and saturated fat (Mahley, 1979; Havel, 1982; Brewer *et al.*, 1983). Epidemiological studies have established a correlation between the apoE phenotype and alterations in the plasma level of remnant lipoproteins, apoB and LDL cholesterol (Davignan *et al.*, 1988).

Recent studies in non-human primates have demonstrated injury by free radicals as another possible mechanism of initiation of atherogenesis, which was protected by the antioxidant, probucol (Sasahara *et al.*, 1994). The fact that probucol prevents the formation of fatty streaks and suppresses the inflammatory processes required for lesion development bears evidence to this effect. These studies have provided new insights into the role of oxidation and other chemical modifications of LDL, such as glycation in the process of atherogenesis. In particular, the oxidative modification of LDL enhances its atherogenecity (Steinberg, 1988; Duthie *et al.*, 1989; Palinski *et al.*, 1989). Recent studies have demonstrated that oxidized lipids, especially oxidized fatty acids and cholesterol in the diet are atherogenic (Staprans *et al.*, 1998).

Thus, even though oxidative metabolism clearly has many advantages, but free radicals generated through this process are highly reactive and can damage innocent bystander molecules, such as DNA, proteins, lipids, and carbohydrates (Southorn and Povis, 1988). The unpaired electron in the outer shell of free radicals is in a high energy state and determines its high reactivity. Oxygen free radicals found in biological systems include the superoxides, hydroxyl, and hydroperoxyl radical. Hydrogen peroxide though not in itself a free radical, easily produces oxygen free radicals. Singlet oxygen is another highly reactive species that gets transformed into free radical by absorption of a single photon. These oxygen free radicals will oxidize any nearby molecule, such as fatty acid, in an

attempt to shed the high energy electron. Free radicals initiate lipid peroxidation, a chain reaction resulting in the formation of a new fatty acid free radical which will in turn attack another fatty acid and in the process transform it into a lipid hydroperoxide. This propagation reaction initiated by a single free radical results in damage to thousands of fatty acid molecules. Free radical reactions terminate when two free radicals meet and combine in such a way that their unpaired electrons bind together.

As humans evolved to use oxidative metabolism, many mechanisms have been developed to control this process and minimize random free radical oxidation. Firstly oxidative metabolism is compartmentalized for example, in the mitochondria. Secondly molecular oxygen and its reactive free radical species are tightly bound to enzymes, as in case of the cytochrome systems during oxidative phosphorylation. Thirdly in order to prevent free radical formation, transition metals like copper and iron which in the free form catalyze free radical formation, are tightly bound to transport and storage proteins. Fourthly several enzymes exist within the cells to neutralize free radicals. Superoxide dismutase catalyses the transformation of superoxide radicals to hydrogen peroxide. Similarly, catalase and glutathione dismutase neutralize hydrogen peroxide and fatty acid radicals. Finally damage caused by oxidation can be repaired by specific enzymes.

If, however, inspite of the availability of all the above mentioned protective mechanisms against oxidative damage, due to any reason, the free radical peroxidation of LDL lipids occurs, it results in numerous structural changes, all depending on a common initiating event i.e., peroxidation of polyunsaturated fatty acids on LDL (Jurjens *et al.*, 1987; Steinbrecher *et al.*, 1990). Also, all the major cells of arterial wall (endothelial cells, macrophages, smooth muscle cells) can oxidatively modify LDL. Both forms of oxidised LDL are taken up more avidly by the macrophages than the LDL itself and this aviditiy appears to be facilitated by

scavenger receptor mechanism (Jurjens *et al.*, 1987; Steinbrecher *et al.*, 1990). The biological effects of oxidised LDL reported to date could contribute to initiation and progression of atherosclerotic process. Oxidised and partially oxidised LDL itself or the oxidised products derived from it can have a number of deleterious effects, including chemotaxis for monocytes (Qureshi *et al.*, 1991), inhibition of nitric oxide mediated relaxation of coronary tone (Schaefer *et al.*, 1995). To summarise, recognition of uncontrolled uptake of oxidised LDL by macrophages leads to a chain of biochemical events which can result in the formation of foam cells, fatty streaks and later atherosclerosis.

Pharmacological control over cholesterol biosynthesis has long been sought as a means for regulating the amount of cholesterol in the blood and for the prevention and treatment of atherosclerosis (Kolata, 1983). Probucol has been reported to effectively reduce plasma cholesterol in human and a number of animal species (Miettinen and Toinonen, 1975; Martz, 1979; Sunson *et al.*, 1981). It also affects the composition and *in vitro* catabolism of LDL in Type IIa hypercholesterolemia (Baudet *et al.*, 1986). It increases the activity of plasma lipoprotein lipase and decrease HDL- and LDL-cholesterol concentration in rats. Probucol prevents the development of macrophages into foam cells by inhibiting the lipid storage in macrophages (Yamamoto *et al.*, 1986a). These observations probably accounts for the clinical findings that probucol causes a more marked regression of xanthomas than would be expected from the of lowering of LDL-cholesterol levels alone. Probucol seems to act by increasing LDL removal from the plasma by an LDL receptor independent mechanism (Kasaniemi and Grundy, 1984), as it causes moderate reduction in LDL-cholesterol in non-familial hypercholesterolemia (Sunson *et al.*, 1981) and a smaller decrease in familial hypercholesterolemic patients (Durrington and Miller, 1985; Fellin, 1986). A marked decrease in cholesterol has been constant finding (Kasaniemi and Grundy, 1984; Fellin, 1986)

but circulating HDL-cholesterol in probucol treated patients is less than in controls (Yamamoto *et al.*, 1986b), which minimizes the use of probucol as a hypocholesterolemic agent.

Fibrates are orally active compounds with relative long plasma half life. Among fibrates, clofibrates, with combined maximal effectiveness and minimal toxicity in the initial screen, was widely used in the management of hyperlipoproteinemia in humans. More recently, however, due to questionable activity in the secondary prevention of atherosclerosis (Coronary Drug Project, 1975) and to the observation of untoward effects in primary prevention (Committee of Principal Investigators, 1978), the use of clofibrates has become limited. Among side effects, proliferation of peroxisomes in rodents has been a key target for chemical and pharmacological studies. A number of clinical trials have constantly shown that clofibrates reduces plasma TAG levels substantially, affecting both VLDL and LDL associated TAG. This reduction is more evident in hypertriglyceridemic patients but less marked in normotriglyceridemic subjects (Hunninghake *et al.*, 1981; Crouse and Grundy, 1981).

Halofenate, structurally related to clofibrate, is hypolipidemic and hypourecemic (Sirtory *et al.*, 1972) and is effective in lowering serum TAG in rats (Kovanen *et al.*, 1981). Bezafibrate, a fibric acid derivative, has been reported to lower plasma TAG in hypertriglyceridemic patients (Eisenberg *et al.*, 1984) and was effective in reversing most if not all, of the abnormalities in lipoprotein compositions, structure and function detected in these patients. Fenofibrate, another analogue, is more potent than the previously described compounds, being fully active at a daily dose of 300 mg (Rossner and Oro, 1981). It is markedly effective in patients with type II and IV hyperlipidemias (Franceschini *et al.*, 1985) as well as in subjects with familial combined hyperlipidemia (Weisweiler *et al.*, 1984) in decreasing total plasma cholesterol and TAG levels. However, HDL-

cholesterol levels were unchanged in type II and combined hyperlipidemia (Malmendier and Delcroise, 1985). The mechanism of hypolipidemic effect of fenofibrate is different from that of parent compound involving its effect on lipoproteins as seen in subjects with familial hypercholesterolemia as well as combined hyperlipidemia.

Nicotinic acid is possibly the oldest lipid lowering drug. Large doses of this drug rapidly reduce plasma TAG by lowering VLDL in normal and sucrose diet fed mice (Oliver *et al.*, 1988). The reduction in TAG was reported to be due to decreased production of VLDL (Grundy *et al.*, 1981). To overcome the difficulty of obtaining adequate compliance with nicotinic acid treatment, because of drug's numerous side effects. several analogues and derivatives have been tested but no clear advantages have been gained over the parent drug (Cretaldi *et al.*, 1988).

An alternate approach to the control of cholesterol level is the use of bile sequestrant resin, such as cholestyramine. The mode of action of bile acid binding resin is apparently fairly simple: bile acids, which are bound to these resins in the intestinal lumen are not reabsorbed, and are excreted with faeces. These sequestrant agents thus interfere with the enterohepatic circulation of bile acids, and since bile acids are synthesized in the liver from cholesterol, cholesterol catabolism is enhanced. After prolonged treatment hypocholesterolemia is observed. Homozygous type II patients are insensitive to cholestyramine inspite of increased level of fecal bile acids (Moutafis *et al.*, 1971). The LDL pathway receptor plays a role in the action of bile acid binding resin (Shepherd *et al.*, 1980).

An encouraging development in the treatment of hypercholesterolemia has been the introduction of a new class of fungal-derived compounds that are potent competitive inhibitors of HMG-CoA reductase. These drugs are extremely

effective in lowering plasma concentrations of LDL-cholesterol. The development of these specific inhibitors of HMG-CoA reductase has considerably widened the therapeutic opportunities in hypercholesterolemic patients. Compactin and lovastatin (mevinolin) are potent competitive inhibitors of HMG-CoA reductase. The molecular structure closely resemble the HMG moiety of HMG-CoA, and the enzyme HMG-CoA reductase binds both the compounds with high affinity (Paolletti and Poli, 1987). These drugs have been used to reduce plasma cholesterol levels in many animal species (Alberts *et al.*, 1980; Kovanen *et al.*, 1981; Tobert *et al.*, 1982). In clinical studies, lovastatin and compactin effectively reduce plasma LDL in normal (Tobert *et al.*, 1982) as well as in subjects with heterozygous familial hypercholesterolemia (Bilheimer *et al.*, 1983). A compensatory increase in the receptor mediated catabolism of LDL also occurs (Grundy and Bilheimer, 1984). In patients with type IIa and type IIb hypercholesterolemia, reduction in serum cholesterol was reported after lovastatin treatment. Goldstein and Brown (1984a) proposed that statins (compactin, lovastatin and simvastatin) lower plasma LDL cholesterol by inhibiting cholesterol biosynthesis and increasing the number of LDL receptors. Although an increase in HDL levels has also been reported in several studies (Hoeg *et al.*, 1986; Mol *et al.*, 1988), others (Mabuchi *et al.*, 1981; Illingworth and Sextan, 1984) have found no change in HDL levels.

The mechanism of action of lovastatin differs from that of probucol (Helve and Tikkanen, 1988). Lovastatin therapy resulted in the increase of both HDL and HDL_2-cholesterol whereas with probucol HDL-cholesterol was markedly decreased mainly because of reduction in HDL_2. Triacylglycerols remain unaltered during lovastatin treatment and no significant changes in lipase activity was observed indicating that these enzymes were not involved in its action.

3-Hydroxy-3-methylglutaric acid (HMG), which is formed by the hydrolysis of HMG-CoA catalysed by HMG-CoA hydrolase in liver, is also known to inhibit cholesterogenesis between HMG-CoA and mevalonate (Rabinowitz and Gurin, 1954) and competitively inhibit the enzyme HMG-CoA reductase (Fimognari and Rodwell, 1965). The hypolipidemic activity of HMG has been studied in rats (Yousufzai and Siddiqi, 1976a, 1977; Francesconi *et al.*, 1987), rabbits (Lupien *et al.*, 1973), hamster (Padova *et al.*, 1982) and humans (Lupien *et al.*, 1973; Yousufzai and siddiqui, 1976b). A support to the hypolipidemic action of HMG is also obtained from the observation that low incidence of CHD in *Massai* tribesman is related to their high consumption of cow's milk (Richardson, 1978), known to contain HMG (Mann, 1977).

Inhibitors of HMG-CoA reductase block synthesis of cholesterol in the liver, thereby triggering compensatory reactions that lead to a reduction in plasma LDL. Much of the information about the mechanism for this reduction comes from studies in cell culture and in experimental animals (Groot *et al.*, 1992). Cultured human fibroblasts respond to an inhibition of HMG-CoA reductase by accumulating increased amounts of the enzyme (Brown *et al.*, 1978). The increase is attributable to an increase in the rate of transcription of the HMG-CoA reductase gene, an increase in the rate of translation of the mRNA, and a decrease in the rate of degradation of the protein. Through these compensatory mechanisms, cultured cells can increase the amount of HMG-CoA reductase sufficiently to restore rates of cholesterol synthesis almost to normal, even in the presence of relatively high concentrations of the inhibitor. An increase in HMG-CoA reductase also occurs in the livers of rabbits, hamsters, and rats (Endo *et al.*, 1979; Tanaka *et al.*, 1982) treated with these inhibitors. A similar adaptation is presumed to occur in humans. Hamster has been reported to respond to hypocholesterolemic drugs (Suckling *et al.*, 1991) and develop atherosclerosis under appropriate conditions

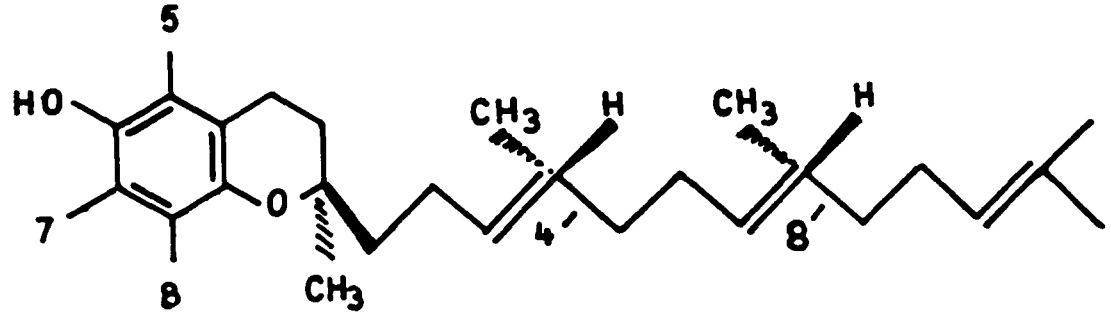
(Nistor *et al.*, 1987; Sima *et al.*, 1990), although differences between the hamster and man which hamper the use of this species as a model for human atherosclerosis have been noted (Nikkari *et al.*, 1991). Rats also respond to hypocholesterolemic drugs but in a different way to hamsters. Rats are very capable of modulating their hepatic cholesterol synthesis and this is the first response to drugs such as bile acid sequestrants and HMG-CoA reductase inhibitors. However, hyperlipidemia can be induced and in some cases this can lead to atherosclerosis (Russell *et al.*, 1990; Vance and Russell, 1990; Russell *et al.*, 1991). Inhibition of mevalonate and cholesterol synthesis by inhibitors of HMG-CoA reductase such as mevastatin and lovastatin prevents farnesylation and blocks cells growth (Maltese and Robishaw, 1990). It has been postulated that inhibitors of HMG-CoA reductase such as lovastatin used in the treatment of hypercholesterolemia, may also be useful for cancer chemotherapy (Schafer *et al.*, 1989). HMG-CoA reductase inhibitors and/or inhibitors of Ras farnesyl : protein transferase could prevent farnesylation of Ras proteins, inhibiting the growth of Ras-dependent tumour cells (Goldstein and Brown, 1990). Lovastatin and sodium phenylacetate, an inhibitor of isopentenyl pyrophosphate synthesis are in clinical trials for the treatment of malignant gliomas (Shack *et al.*, 1994).

One of the areas which has attracted a great deal of attention is antioxidant nutrition in the control of degenerative diseases such as atherosclerosis and cancer (Ames, 1983; Ong and Packer, 1992). Peroxidation of the cellular membrane lipids is a basic reaction which results in the deterioration of unsaturated fatty acids in the membrane. This process has been implicated in several human diseases and in the toxicity of xenobiotics (Yagi, 1982; Halliwell and Gutteridge, 1989; Sies, 1991). If lipid peroxidation is triggered, it can inactivate cellular components and its products can have serious consequences on almost all the crucial molecules leading to diseased conditions (Halliwell and Gutteridge, 1989; Sies, 1991). The

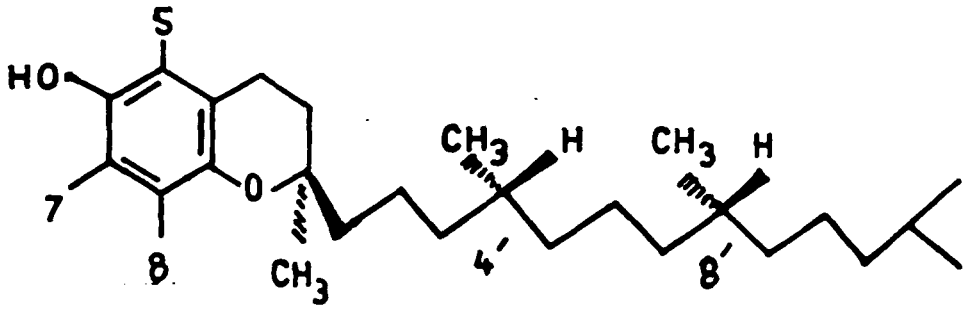
peroxidation products can also cause the formation of 8-hydroxydeoxyguanosine whose presence in the genetic material can lead to mutagenesis and carcinogenesis (Kuchino *et al.*, 1987; Park and Floyd, 1992). The susceptibility of tissues to lipid peroxidation is influenced by the lipid and antioxidant composition of cellular membranes which in turn may be controlled by dietary composition (Clegg, 1973; Manorama and Rukmini, 1992).

The greater stability of vegetable oils versus animal fats under oxidative conditions is known to be due to the higher levels of natural antioxidants in the oils. An important and commonly occurring class of natural antioxidants in vegetable oils is tocopherols (T) of vitamin E family. There are 8 naturally occurring forms of vitamin E; α-, β-, γ-, δ-tocopherols and tocotrienols (T_3). Tocotrienols are minor plant constituents especially abundant in cereal grains (such as barley, oat, wheat and rye), rice bran, palm oil and latex (Kasparek, 1980b). The vitamin E antioxidant property reflects the similarity in chemical structures of T and T_3, which differ only in possessing a farnesyl or unsaturated phytyl side chain, respectively (Kasparek, 1980a) (Fig. 1.5). Tocopherols predominate in certain oils such as corn oil, soyabean oil and olive oil. Whereas, the T_3 series predominates in rice bran oil (RBO), palm oil and barley oil. Small amounts of T_3 are found in carrots, sweetcorn and germ oils (Shin and Godber, 1994). Several lines of research have established that populations which consume large amounts of cereal grain and vegetable oils tend to have a lower incidence of cardiovascular disease (Sacks *et al.*, 1975; Burslem *et al.*, 1978; Gould *et al.*, 1980). Furthermore, studies on cereal grains demonstrated that barley is particularly effective in lowering lipid levels in animal models (Qureshi *et al.*, 1980 a,b&c). The ability of barley extracts to lower lipids *in vivo* led to the purification and identification of biologically active compound tocotrienol (Qureshi *et al.*, 1986). There are scattered reports that neither rice bran nor RBO

Tocotrienol



Tocopherol



Position of methyl group	Tocotrienols	Tocopherols
5,7,8-Trimethyl	α-T_3	α-T
5,8-Dimethyl	β-T_3	β-T
7,8-Dimethyl	γ-T_3	γ-T
8-Monomethyl	δ-T_3	δ-T

Fig. 1.5. Structures of tocotrienols and tocopherols.

lowered cholesterol levels. These findings may be explained by reports that some, but not all, rice cultivars contain tocotrienols which exert a powerful hypocholesterolemic action (Qureshi *et al.*, 1986; Qureshi *et al.*, 1989). Qureshi *et al.* (1986) have demonstrated the hypocholesterolemic effect of tocotrienols isolated from barley, oats, rice and palm oil in various animal models. Anticholesterol impact of tocotrienols has also been demonstrated in hypercholesterolemic subjects (Qureshi *et al.*, 1995). Out of α-, β-, γ- and δ-T_3, γ and δ-T_3 have been found to be most potent in terms of their HMG-CoA reductase inhibition as well as cholesterol lowering effects. The efficiency of hypocholesterolemic action as well as the degree of inhibition of HMG-CoA reductase activity mediated by α-T_3 was substantially lower than γ and δ- T_3 (Pearce *et al.*, 1992). β-form of T_3 failed to exhibit any anticholesterol activity. Rice bran oil is the richest source of T_3, whereas corn, groundnut, mustard, soyabean and coconut oils and butter fat contain only T, which have no lipid lowering effect. The T_3 are highly effective in lowering total blood cholesterol and LDL-cholesterol apparently by reducing the HMG-CoA reductase activity. The T on the other hand do not inhibit cholesterol synthesis and thus do not lower serum cholesterol. A dose dependent effect of tocotrienol rich fraction (TRF) isolated from palm oil was observed for lowering the serum cholesterol and LDL-cholesterol in normolipidemic and hypercholesterolemic swine, quail and chicken (Pearce *et al.*, 1992; Qureshi and Qureshi, 1993).

In several respects T_3 appear to operate in similar manner to oxysterols. Certain oxysterols have been shown to regulate cholesterol biosynthesis by transcriptional down-regulation of reductase gene (Kandutsch *et al.*, 1978; Schroepfer *et al.*, 1979; Schroepfer *et al.*, 1981; Schroepfer *et al.*, 1982; Miller *et al.*, 1982). It has been postulated that endogenously produced oxysterols are natural regulators of cholesterol biosynthesis. These oxysterols are potent

repressors of HMG-CoA reductase and bind strongly to cytosolic oxysterol binding protein (Spencer *et al.*, 1985; Saucier *et al.*, 1985). Since oxysterols, are natural regulators of cholesterol biosynthesis and acts by suppressing HMG-CoA reductase gene, the T_3 may have a similar function, but it acts at post-transcriptional level as has been experimentally demonstrated in HepG2 cells (Parker *et al.*, 1993).

The human hepatoma HepG2 cell culture model was employed to compare the intrinsic activities of T_3. In HepG2 cells, inhibition of sterol synthesis correlate with rapid suppression of HMG-CoA reductase when incubated with T_3. The recemic synthetic tocotrienols exhibit comparable biological activity to the natural tocotrienols in the cholesterol suppression activity. Gamma-Tocotrienol has been shown to mediate the suppression of enzymatic activity and protein mass of HMG-CoA reductase in HepG2 cells, through decreased synthesis (57% of control) and enhanced degradation (2.4 fold versus control) of the enzyme (Parker *et al.*, 1993). Thus, tocotrienols influence the mevalonate pathway in mammalian cells *in vitro*, by post-transcriptional suppression of HMG-CoA reductase, and appear to specifically modulate the intracellular mechanism for controlled degradation of the reductase protein (Parker *et al.*, 1993). These activities of tocotrienols in HepG2 cells mirror the actions of the putative non-sterol feedback regulators derived from mevalonate in cultured cells (Goldstein and Brown, 1990). In contrast to the effects of 25-hydroxycholesterol, T_3 does not suppress LDL receptor protein in HepG2 cell membranes as demonstrated by western blotting.

Since the discovery of vitamin E, in Berkeley by H.M. Evans in 1992, when it was first described as antisterility agent, many scientists and physicians have sought to elucidate its biochemistry, health benefits and clinical applications (Lester, 1992). Vitamin E has been well accepted as natures most effective lipid soluble, chain-breaking antioxidant. It is the first line of defense against lipid

peroxidation protecting polyunsaturated fatty acids in cell membranes through its free radical quenching activity in biomembranes at an early stage of free radical attack (Horwitt, 1986). After being oxidized in this process and before being decomposed, vitamin E can be re-reduced by ascorbic acid and glutathione. This reaction is dependent on the concentration of these substances and/or the enzymes that maintain them in their reduced form.

The antioxidant activity of T and T_3 is mainly due to their ability to donate their phenolic hydrogens to lipid free radicals (Burton and Ingold, 1986; Pokorny, 1987; Burton and Ingold, 1988; Burton and Traber, 1990). The mechanism for the inhibition of lipid peroxidation in biological membranes by T and T_3 can be outlined as follows: The peroxidation proceeds in 3 phases; initiation, propagation and termination (Burton and Traber, 1990). In the initiation phase a carbon-centered lipid radical $R^{\bullet}$ is produced from a polyunsaturated fatty acid RH. This $R^{\bullet}$ reacts with molecular oxygen in the propagation phase to form $ROO^{\bullet}$, which reacts with other RH forming a hydroperoxide, ROOH.

$$R^{\bullet} + O2 \rightarrow ROO^{\bullet}$$

$$ROO^{\bullet} + RH \rightarrow ROOH + R^{\bullet}$$

This propagation process continues and consumes the valuable polyunsaturated fatty acids. In the termination phase, the chain reaction stops when a peroxyl radical ($ROO^{\bullet}$) combines with another $ROO^{\bullet}$.

$$ROO^{\bullet} + ROO^{\bullet} \rightarrow \text{Inactive product}$$

However, tocotrienols (T_3-OH) and tocopherols (T-OH) can intercept the peroxyl radical more rapidly than can polyunsaturated fatty acids by the following reactions:

$$T_3\text{-}OH + ROO^{\bullet} \rightarrow T_3\text{-}O^{\bullet} + ROOH$$

$$T_3\text{-}O^{\bullet} + ROO^{\bullet} \rightarrow \text{Inactive product}$$

The T_3-$O^{\bullet}$ radical is unable to continue the chain and reacts with the peroxyl to form inactive product.

Although, it is generally agreed that the relative antioxidant activity of T *in vivo* is in the order of $\alpha>\beta>\gamma>\delta$ (Dillard *et al.*, 1983; Burton and Ingold, 1986; Burton and Traber, 1990; VERIS, 1993), there is a wide spread confusion concerning their relative potency *in vitro* (Burton and Ingold, 1981). The chemical structures of T and T_3 support hydrogen-donating power in the order of $\alpha>\beta>\gamma>\delta$ (Pokorny, 1987). This order also was obtained when the activity of the four T was compared in a homogenous solution in dichlorobenzene (Burton and Ingold, 1981), but a reverse order ($\delta>\gamma\cong\beta>\alpha$) was obtained when the relative antioxidant properties were compared in fats, oils and lipoproteins *in vitro* (Lea and Ward, 1959; Olcott and Van Der Ven, 1968; Parkhurst *et al.*, 1968; Koskas *et al.*, 1984; Esterbauer *et al.*, 1989; Gottstein and Grosch, 1990). The reason behind this order is not yet clearly understood. Several studies have demonstrated the ability of tocopherols to prevent *ex vivo* and *in vitro* LDL-C oxidation and significantly reduce the development of atherosclerotic lesions (Carew *et al.*, 1987; Esterbauer *et al.*, 1991; Mao *et al.*, 1991), risk of coronary heart disease (CHD) (Rimm *et al.*, 1993; Stampfer *et al.*, 1993) and ischemic heart disease (Gey, 1995). Even though, α-T has been labeled as the most efficient chain-breaking antioxidant, tocotrienols are known to be more potent antioxidant than tocopherols (Suarna *et al.*, 1993; Kamat and Devasagayam, 1995; Kamat *et al.*, 1997). Tocotrienols have been shown to have greater free radical scavenging properties as cell membrane constituents than T (Yamaoka and Carrillo, 1990; Serbinova *et al.*, 1991). Recently, it was shown that compared to α-T, α-T_3 possesses a 40-60 times higher antioxidant activity against Fe^{2+} + ascorbate- and Fe^{2+} + NADPH-induced lipid peroxidation in rat liver microsomal membranes and 6.5 times greater protection of

cytochrome P-450 against oxidative damage (Serbinova *et al.*, 1991). This higher antioxidant potency of α-T_3 as compared to α-T is attributed to the combined effects of three properties; its higher recycling efficiency from chromanoxyl radical, its more uniform distribution in membrane bilayer, and its stronger disordering of membrane lipids which makes interaction of chromanols with lipid radicals more efficient.

Several animal studies have been conducted to test the hypothesis that antioxidants delay atherosclerosis. Wojcicki reported a statistically significant 25 per cent reduction in aortic atherosclerotic lesions in hypercholesterolemic mongrel rabbits fed 10 mg/kg/day of vitamin E compared to controls (Wojcicki *et al.*, 1991) and Verlangieri reported a 54 per cent lesion reduction in monkeys fed 108 IU of vitamin E per day compared to controls (Verlangieri and Bush, 1992). Qureshi *et al.* (1991 a&b) have reported a reduction in both total and LDL-cholesterol in human hyperlipidemics and pigs supplemented with T_3. Supplementation of γ-T_3 along with an atherogenic diet to rats for six weeks have been shown to lead to decreased plasma lipid and lipoprotein concentrations. In addition, a decrease in the plasma lipid peroxidation was shown (Watkins *et al.*, 1993). Similar observations were reported in hypercholesterolemic rabbits by Teoh *et al.* (1994). Wahlquist *et al.* (1992), however, observed a differential response to T and T_3 supplementation in human subjects without any change in serum lipids. Tomeo *et al.* (1995) also reported that T_3 significantly decreased plasma lipid peroxidation in patients with hyperlipidemia and carotid stenosis with no change in their lipid and lipoprotein parameters. Similarly, Mensink *et al.* (1999) recently reported that T_3 had no markedly favourable effects on the serum lipoprotein profile in men with slightly elevated lipid concentrations. Experiments involving the effects of T_3 on apoB (LDL) and apoA-I (HDL) and apoB/apoA-I ratio has been reported in chickens, swine and humans. The apoB/apoA-I ratio, considered to be a better

indicator than LDL/HDL ratio, for assessment of CHD, was reduced in T_3 treated subjects (Qureshi *et al.*, 1991 a&b). Recently, the mechanism of action of T_3 on apoB metabolism in HepG2 cells has been investigated (Wang *et al.*, 1998; Theriault *et al.*, 1999b).

For a long time now it has been shown that dietary regimen in conjunction with exercise favourably alter the lipid parameters in hypercholesterolemic individuals. Based on the current knowledge dietary recommendations to optimize plasma lipid profiles and thus reduce the risk of coronary heart disease are currently focused on the total fat content, the fatty acid profile, and the cholesterol content of the diet (Dietary Guidelines, 1988). Public health programmes for prevention of CAD in developed countries recommend changes in dietary habit and patterns of various components of diets, especially dietary fat. Recently a reduction in dietary intake of total fat, particularly animal fat with a limited amount of saturated fatty acid and polyunsaturated fatty acid, has been suggested (Lesserre *et al.*, 1985). The American Heart Association (1982) recommends that total fat content of the diet should not exceed 30% of total calories, saturated fatty acid should not exceed 10%, monounsaturated fatty acid should not exceed 10% and polyunsaturated fatty acid should not exceed 10% of calories. Lasserre *et al.* (1985) reported that linolenic acid (18:3) should provide 0.5-1.0% of total calories.

Polyunsaturated fatty acids are abundantly available in vegetable oils like safflower oil, sunflower oil and corn oil which were found to lower blood total and LDL-cholesterol levels, as revealed by epidemiological studies (Berkson and Stamler, 1981). A number of studies in humans and animals have shown that RBO is as effective as other vegetable oils in lowering plasma cholesterol levels (Rukmini and Raghuram, 1991; Lichtenstein *et al.*, 1994). In some cases, RBO lowered plasma cholesterol more effectively than other commonly used vegetable oils rich in linoleic acid (Rukmini and Raghuram, 1991); this effect was attributed

to the presence of T and T_3 in the RBO (Nicolosi *et al.*, 1991). However, Qureshi *et al.* (1991a & b), confirmed the impact of tocotrienols on cholesterol, specifically the LDL-cholesterol, in animal and human studies. The tocopherols on the other hand do not lower serum cholesterol (Qureshi *et al.*, 1989). Recently, biological properties of tocotrienols such as hypolipidemic, antioxidant and anti-tumour, have been reviewed in detail (Theriault *et al.*, 1999a).

Rice is one of the foremost food crops of the world. It is staple food for more than half of the worlds population. More than 90% of the worlds rice production is concentrated in Asia, especially in China, India, Indonesia, Bangladesh, Thailand, Burma and Japan. India is the largest producer of rice in the world outside mainland China. The total paddy production in 1996 was 120 million tons, whereas projected paddy production in India for year 2000 is expected to be 1440 million tons. The rice bran availability for 1996 was 60 million tons (at the rate of 7.5% of rice) and projected bran production for the year 2000 is 70 million tons (Food and Civil Supplies Department, NewDelhi, India, 1996). Rice bran refers to the coating removed from the brown rice during the process of milling. The bran constitutes nearly 8.5-10% of the total grains and is highly nutritious. It is rich in lipids, proteins, minerals and vitamins. Rice bran is most valuable by product of rice milling industry. It contains 12-15% proteins, 14-20% oil if paddy is raw and 18-25% if paddy is parboiled. About 60% of paddy produced in India is parboiled. Although the US produces only 1-2% share of the total world rice crop, it is a major exporter having a 20-30%, share of the world export trade (Migura, 1989). Rice bran processed in the US is a potential source of over 100,000 metric tons of tocotrienol rich oil. In the last 10 to 15 years US scientists have shown interest in the cholesterol lowering property of rice bran (Haumanh, 1989) and rice bran products have been introduced in supermarkets.

Rice bran oil has the potential of being the oil of choice for health-conscious consumers in the US.

Rice bran oil, though not a popular edible oil worldwide, is in steady demand as the so called *healthy oil* not only in Japan but also in other Asian countries, particularly India. Only 0.5 million ton of RBO corresponding to 8.5% of total vegetable oils is consumed annually in India (Central Organisation for Oil Industry and Trade, India, 1998). Similarly, 1000 ton of RBO corresponding to 3.5% of total vegetable oils is consumed in Japan. Further RBO is characterized by its relatively high content of unsaponifiable material. The content of the unsaponifiable material in refined edible grade RBO is less than 5% under Japan Agricultural Standards. This value of non-saponifiable fraction of RBO is considerably higher than that of other vegetable oils including palm oil where it is less than 1-1.5%.

Rice bran oil, therefore, has the potential of being the oil of choice for population with a high risk of CAD due to increase in plasma lipid levels. Rice bran oil as an edible oil may be one of the healthiest and safest oils for human nutrition. The use of RBO or other commonly used edible oil supplemented with optimal concentration of T_3 by normal population will prevent the occurrence of hypercholesterolemia and cardiovascular diseases. Furthermore, daily consumption of RBO as cooking oil by subjects suffering from lipid abnormalities will be useful in the treatment of hypercholesterolemia and coronary heart disease.

1.1 Objectives of the present investigation

Current worldwide interest in the use of tocotrienols (T_3) in the prevention and treatment of cardiovascular diseases, creates a need to develop a simple and cost-effective methodology for the isolation of tocotrienol and tocopherol rich fraction (TRF) from rice bran/rice bran oil (RBO). Since, a systematic and detailed investigation related to hypolipidemic and antioxidant properties of TRF isolated from RBO, in normolipidemic and hyperlipidemic rats has been lacking, we have undertaken a detailed investigation pertaining to hypolipidemic and antioxidant impacts, including elucidation of possible mechanism of cholesterol lowering action of TRF in normolipidemic and hyperlipidemic rats. In addition, due to the availability of a rare familial hypercholesterolemic (FH) patient with severe rapidly growing skin xanthomas, we investigated the impact of TRF on cholesterol dynamics of this patient. Hypolipidemic property of RBO, rich in tocotrienols was also investigated in normolipidemic humans.

The efficacy of TRF in the prevention of experimentally-induced hyperlipidemia in rats was investigated by administration of TRF along with atherogenic diet. In addition, efficiency of TRF in the treatment of hyperlipidemia after the withdrawal of atherogenic diet was also investigated in hyperlipidemic rats. The minimum dose of TRF required to exert the maximum hypolipidemic effect in normolipidemic and hyperlipidemic rats was also determined.

There are scattered reports showing the varying hypocholesterolemic activity of rice bran or rice bran oil (RBO) and some have shown that neither rice bran nor RBO lowered cholesterol levels. These findings may be explained by other reports that some, but not all, rice cultivars contain tocotrienols (T_3), which exert a powerful hypocholesterolemic action. Because of these variation in types and content of T_3, one finds variation in the cholesterol lowering effects of different

RBO isolated from different cultivars of rice. Out of α-, β-, γ- and δ-T_3, γ- and δ-T_3. were the most potent in terms of cholesterol lowering effect, α-T_3 has very low effect, whereas, β-form has no anti-cholesterol activity. Therefore, we have investigated the hypolipidemic potency of TRF isolated from RBO of two rice cultivars, Basmati and Saket-4, based on their γ- and δ-T_3 content.

Earlier reports indicate that α-, γ- and δ-T_3, isolated from palm oil, exert their *in vivo* hypocholesterolemic effect in chickens, by reducing the HMG-CoA reductase activity. In another report in HepG2 cells, T_3 have been shown to suppress cholesterol formation by reducing the enzymatic activity of HMG-CoA reductase. This reduction in enzymatic activity was due to decrease in HMG-CoA reductase protein mass, involving its enhanced degradation at post-transcriptional level. However, the mechanism of T_3-mediated *in vivo* regulation of HMG-CoA reductase activity has not been reported. In the present study we have investigated the possible mechanism of lipid lowering action of tocotrienols (TRF) in normolipidemic and hyperlipidemic rats. This objective was achieved by quantifying the enzymatic activity of HMG-CoA reductase and its immuno-reactive protein mass in the liver of normolipidemic and hyperlipidemic rats treated with TRF.

Published reports indicate that oxidized-LDL is more atherogenic than native-LDL and over-production of free radicals is linked with acceleration of atherosclerotic process. Tocotrienols, in comparison to tocopherols, have been shown to exert more potent antioxidant activity *in vitro* and *ex vivo* in hepatic microsomes and plasma LDL of normal rats. Therefore, *in vivo* impact of TRF, as antioxidant, in the prevention of lipid peroxidation and plasma LDL oxidation was investigated in normolipidemic and experimentally induced hyperlipidemic rats.

In familial hypercholesterolemic patients an elevated plasma total and LDL-cholesterol and high levels of apoB are major risk factors in the development of coronary heart disease (CHD). Numerous studies have also demonstrated an inverse relationship between plasma levels of HDL-C and CHD incidence and coronary atherosclerosis. Case-control studies indicate that the inverse relationship with CHD is mainly due to HDL subfraction, HDL_2-C, which is the strongest predictor of the both presence and extent of CAD. Intervention studies with repeated angiographics to monitor disease progression have consistently shown that HDL-C and its apoA-1 and LDL-C and its apoB concentrations or the TC to HDL-C ratio are independently associated with growth of atherosclerotic lesions. The most effective approach for controlling FH is by inhibiting cholesterol synthesis at the level of HMG-CoA reductase, the rate limiting enzyme. Therefore, based on known inhibitory action of TRF on hepatic HMG-CoA reductase activity, we have investigated the impacts of TRF on plasma trigycerides, total cholesterol, VLDL-C and LDL-C including apoB, in a 5 year-old FH boy with rapidly growing xanthomas on buttocks extending to thighs, elbows and knee. Since, HDL-C sub-fraction, HDL_2-C has been shown to be a strongest predictor of both presence and extent of CAD in humans, the impact of TRF on plasma HDL-C including apoA-1 and its sub-fractions, HDL_2-C and HDL_3-C, was also investigated. In addition, the long-term impact of TRF on the growth and size of skin xanthomas was investigated.

Since earlier reports indicate a similar hypolipidemic effects of both RBO and TRF in hyperlipidemic subjects, we have investigated the impact of dietary RBO on plasma lipids of normolipidemic human volunteers.

Experimental

MATERIALS

2.1.1 Chemicals

The chemicals were obtained from various sources listed below.

Chemical		*Source*
Bovine Serum Albumin	-	Miles Laboratories Inc., USA
Cholesterol	-	Sigma Chemical Co., USA
Cholic Acid	-	CDH, India
Commassie Brilliant Blue G250	-	Polysciences Inc., USA
Digitonin	-	Sigma Chemical Co., USA
Ferric Chloride	-	Loba Chemie, India
Glycine	-	SRL, India
Heparin	-	Sigma Chemical Co., USA
Hexane for HPLC	-	Spectrochem. Pvt. Ltd., India
HMG-CoA	-	Pharmacia, Sweden
HRP-conjugate	-	Genei, Bangalore, India
TMB H_2O_2-Substrate	-	Genei, Bangalore, India
Isopropanol for HPLC	-	Merck, Germany
Methanol for HPLC	-	SD Fine Chem. Ltd., India
NADPH	-	Calbiochem, USA
Rat Chow	-	Hindustan Lever Ltd., India
Refined Edible Grade RBO	-	Foods, Fats and Fertilizers Ltd., India
Sodium Dodecyl Sulfate	-	Sigma Chemical Co., USA
Thiobarbutric Acid	-	Sigma Chemical Co., USA
(±)-α-Tocopherol	-	Sigma Chemical Co., USA
Tween-20	-	Sigma Chemical Co., USA

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All other chemicals and reagents used were of analytical grade.

2.1.2 Animals

Male albino rats, weighing about 175-200 g purchased from Central Animal House, J.N. Medical College A.M.U., Aligarh (India), were conditioned to animal house environment prior to the experiment, diet and water was given *ad libitum*. The rats were housed in a light-controlled isolator in which the dark period was maintained from 3.00 a.m. to 3.00 p.m. two weeks before the killing. The rats were sacrificed at sixth hour of dark cycle at the peak of diurnal rhythm of HMG-CoA reductase (Edwards *et al.*, 1977).

2.1.3 Diet

The rats were given pelleted rat chow and water *ad libitum* unless otherwise stated. For the induction of hyperlipidemia, rats were given an atherogenic diet consisting of 5% hydrogenated fat, 0.5% cholic acid and 1% cholesterol. The atherogenic diet, which was given in addition to the pelleted rat chow, was administered as a suspension through gastric intubation in two divided doses (morning and evening) of 0.5 ml each/rat/day. TRF was also administered through gastric intubation in two equal doses of 0.5 ml in saline/rat/day.

METHODS

2.2.1 Isolation and Purification of Tocotrienol Rich Fraction from Crude and Refined Edible Grade Rice Bran Oil

2.2.1.1 Procurement of rice bran and extraction of crude rice bran oil

Raw and parboiled paddies of different cultivars, namely Basmati (long grain) and Saket-4 (short grain) and Mansuri (medium grain) of rice were procured. The brans from these cultivars of rice, representative of different rice growing zones of U.P. (India), were obtained by Sheller milling method. Rice brans obtained from each raw and parboiled paddies of Basmati and Saket-4 were soaked in haxane (7 ml/g bran) within 24 h of milling. The crude rice bran oil (RBO) was extracted in hexane for 72 h with intermittent stirring. The crude RBO fraction was obtained by evaporating hexane under vacuum in a rotary evaporator.

2.2.1.2 Extraction of tocotrienol rich fraction from crude and refined rice bran oil

Tocotrienol rich fraction (TRF) from crude and refined RBO was extracted in methanol by stirring in a container for 1 h. The minimum amount of methanol essential for extraction of maximum amount of TRF had been worked out to be 7 ml per gram of RBO. Methanol layer containing TRF was evaporated at 65 °C under vacuum in a rotary evaporator and crude TRF was recovered.

2.2.1.3 Isolation of purified tocotrienol rich fraction

The procedure for purification of TRF from crude TRF was based on the method reported previously (He *et al.*, 1996) with following modifications. 100 g silica gel (230-400 mesh) was soaked in hexane and layered on a 500 ml scintered glass funnel connected to a water aspirator tube for generating a mild vacuum. The silica gel was washed with 500 ml of hexane and then loaded with 0.5 g of crude TRF in 20 ml of hexane. The gel was washed with 1.5 lit of hexane in order to remove

non-tocotrienol and tocopherol components such as triacylglycerols, sterols, etc. Purified mixtures of tocotrienol (T_3) and tocopherol (T) was then rapidly eluted with diethyl ether. The elution was speeded by the application of vacuum produced by water aspirator. The solvent was evaporated under vacuum in order to obtain purified mixture of T_3 and T. The silica gel was regenerated by thoroughly washing with methanol, which removes all the bound components and reused again for the purification of crude TRF.

2.2.1.4 Analytical HPLC of crude and purified tocotrienol rich fraction

The analytical HPLC system that was used consisted of Beckman System Gold 127 Programmable Solvent Module (127 Pump) and 166 Programmable Detector Module. Samples (20 µl) were injected into Beckman Ultrasphere Silica Column (4.6 mm × 25 cm) using a 20 µl loop. The mobile phase consisted of 0.3% isopropanol in hexane at a flow rate of 1 ml/min and the eluent was monitored at 290 nm by UV detector. The chromatogram obtained were monitored on a HP 3395 integrator. The crude and purified TRF were characterized by comparing the retention time of each peak with the standard TRF. The concentration of individual tocopherols and tocotrienols was calculated from the area under each peak.

2.2.2 Experimental Design

2.2.2.1 Studies in rats

For investigating the hypolipidemic effect of TRF, purified TRF, the experiments were carried out in different sets. Male albino rats were divided in the following groups in each set as described below:

2.2.2.1.1 Experimental Group No. I

⇒ *Normolipidemic Control (NLP-C)*

Five rats were given 0.5 ml saline through gastric intubation for two week.

⇒ *Normolipidemic Treated-1 (NLP-T1)*

Five rats in this group were given 125 mg TRF /kg/day for two weeks.

⇒ *Normolipidemic Treated-2 (NLP-T2)*

Five rats in this group were given 81.25 purified TRF /kg/day for two weeks.

2.2.2.1.2 Experimental Group No. II

⇒ *Normolipidemic Control (NLP-C)*

Three rats were given 0.5 ml saline through gastric intubation for three weeks.

⇒ *Normolipidemic Treated (NLP-T)*

Three rats in this group were given 65 mg purified TRF /kg/day for three weeks.

⇒ *Hyperlipidemic Control (HLP-C)*

Three rats in this group were given an atherogenic diet for three weeks.

⇒ *Hyperlipidemic Treated-1 (HLP-T1)*

Three rats in this group were given 65 mg purified TRF/kg/day alongwith an atherogenic diet for three weeks.

⇒ *Hyperlipidemic Untreated-1 (HLP-U1)*

Three rats in this group were given an atherogenic diet for three weeks and then kept on pelleted rat chow for an additional 5 days.

⇒ *Hyperlipidemic Treated-1 (HLP-T1)*

Three rats in this group, after giving atherogenic diet for three weeks, were given 16.25 mg purified TRF/kg/day for 5 days.

⇒ *Hyperlipidemic Treated-2 (HLP-T2)*

Three rats in this group, after giving atherogenic diet for three weeks, were given 32.50 mg purified TRF/kg/day for 5 days.

⇒ *Hyperlipidemic Untreated-2 (HLP-U2)*

Three rats in this group were given an atherogenic diet for three weeks and then kept on pelleted rat chow for an additional 7 days.

⇒ *Hyperlipidemic Treated-3 (HLP-T3)*

Three rats in this group, after giving atherogenic diet for three weeks, were given 16.25 mg purified TRF/kg/day for 7 days.

⇒ *Hyperlipidemic Treated-4 (HLP-T4)*

Three rats in this group, after giving atherogenic diet for three weeks, were given 32.50 mg purified TRF/kg/day for 7 days.

2.2.2.1.3 Experimental Group No. III

⇒ *Normolipidemic Control (NLP-C)*

Three rats were given 0.5 ml saline through gastric intubation for three weeks.

⇒ *Normolipidemic Treated-1 (NLP-T1)*

Three rats in this group were given 4 mg TRF /kg/day for three weeks.

⇒ *Normolipidemic Treated-2 (NLP-T2)*

Three rats in this group were given 8 mg TRF /kg/day for three weeks.

⇒ *Normolipidemic Treated-3 (NLP-T3)*

Three rats in this group were given 12 mg TRF /kg/day for three weeks.

⇒ *Normolipidemic Treated-4 (NLP-T4)*

Three rats in this group were given 4 mg purified TRF/kg/day for three weeks.

⇒ *Normolipidemic Treated-5 (NLP-T5)*

Three rats in this group were given 8 mg purified TRF/kg/day for three weeks.

⇒ *Normolipidemic Treated-6 (NLP-T6)*

Three rats in this group were given 12 mg purified TRF/kg/day for three weeks.

⇒ *Hyperlipidemic Control (HLP-C)*

Three rats in this group were given an atherogenic diet for three weeks.

⇒ *Hyperlipidemic Untreated (HLP-U)*

Three rats in this group were given an atherogenic diet for three weeks and then kept on pelleted rat chow for an additional 7 days.

⇒ *Hyperlipidemic Treated-1 (HLP-T-1)*

Three rats in this group, after giving atherogenic diet for three weeks, were given 2.6 mg purified TRF/kg/day for 7 days.

⇒ *Hyperlipidemic Treated-2 (HLP-T-2)*

Three rats in this group, after giving atherogenic diet for three weeks, were given 5.2 mg purified TRF/kg/day for 7 days.

⇒ *Hyperlipidemic Treated-3 (HLP-T-3)*

Three rats in this group, after giving atherogenic diet for three weeks, were given 7.8 mg purified TRF/kg/day for 7 days.

2.2.2.1.4 Experimental Group No IV

⇒ *Normolipidemic Control (NLP-C)*

Four rats were given 0.5 ml saline through gastric intubation for three weeks.

⇒ *Hyperlipidemic Control (HLP-C)*

Four rats in this group were given an atherogenic diet for three weeks.

⇒ *Hyperlipidemic Untreated (HLP-U)*

Four rats in this group were given an atherogenic diet for three weeks and then kept on pelleted rat chow for an additional 7 days.

⇒ *Hyperlipidemic Treated-1 (HLP-T-1)*

Four rats in this group, after giving atherogenic diet for three weeks, were given 1.67 mg purified TRF/kg/day from Basmati for 7 days.

⇒ *Hyperlipidemic Treated-2 (HLP-T2)*

Four rats in this group, after giving atherogenic diet for three weeks, were given 3.35 mg purified TRF/kg/day from Basmati for 7 days.

⇒ *Hyperlipidemic Treated-3 (HLP-T3)*

Four rats in this group, after giving atherogenic diet for three weeks, were given 1.17 mg purified TRF/kg/day from Saket-4 for 7 days.

⇒ *Hyperlipidemic Treated-4 (HLP-T4)*

Four rats in this group, after giving atherogenic diet for three weeks, were given 2.34 mg purified TRF/kg/day from Saket-4 for 7 days.

2.2.2.2 *Studies in Human Subjects*

2.2.2.2.1 *Studies in a familial hypercholesterolemic patient*

A 5 year boy presented with a classical case of familial hypercholesterolemia to J.N.Medical college OPD, including a severe rapidly growing skin xanthomas on buttocks extending to thighs, moderately on elbows and couple of them on the knee. Based on above characterization the patient can be categorized as familial hypercholesterolemic. Considering the rare availability of such type of patients, the child was started on low fat, cholesterol free diet, with 8 mg TRF/kg/day for 100 weeks. Ten ml overnight fasted blood sample was collected at the entry point (0 week) and after 8, 20, 60 and 100 weeks of TRF treatment. Plasma was isolated and stored at -20 ^{0}C for analysis.

2.2.2.2.2 *Studies in normolipidemic subjects*

For investigating the hypolipidenic effect of RBO, five apparently healthy female volunteers were involved in the study with an average age of 39.8 ± 12.1 years and average weight of 48.8 ± 9.8 kg. Each volunteer took 0.2 g RBO/kg/day for 4 weeks. At the end of the feeding trial, an interview was conducted to see if the volunteers complied to the feeding schedule. Ten ml overnight fasted blood was taken from each individual at the entry point of the study and after 2 and 4 weeks of RBO intake. Plasma was isolated and stored at -20 ^{0}C for analysis.

2.2.3 Analytical Procedures

2.2.3.1 Collection of blood

At the end of the treatment, the rats in all groups were anaesthetized and blood drawn by cardiac puncture. The blood from each rat in a given group was collected using heparin as anticoagulant. Blood was mixed gently by inversion 2 or 3 times and immediately cooled to 2-4 °C in an ice bath. The samples were centrifuged for 30 min at 2,500 rpm. Plasma was aliquoted in micro-centrifuge tubes and stored at –20 °C.

2.2.3.2 Fractionation of plasma lipoproteins

Lipoproteins were fractionated from plasma by precipitation as described by Kastener (1976). Isolation of HDL-cholesterol and VLDL-cholesterol + LDL-cholesterol was done by using phosphotungstate and magnesium chloride. The following solutions were used:

- 9.7 mM phosphotungstic acid
- 0.4 M magnesium chloride

0.2 volume of 1:1 mixture of phosphotungstic acid and magnesium chloride was added to 1 volume of plasma. The samples were mixed immediately, allowed to stand for 5 min at room temperature and then centrifuged for 10 min at 12,000 rpm at room temperature. The clear supernatant was used for the analysis of HDL-cholesterol as well as for the fractionation of HDL_3-cholesterol and HDL_2-cholesterol. The precipitate was dissolved in 1 volume of freshly prepared 0.1 M sodium citrate and used for the analysis of VLDL-cholesterol + LDL-cholesterol

Fractionation of HDL-cholesterol subfraction and VLDL-cholesterol was done by precipitation method described by Bachorik and Albers (1986). The following solutions were used for the fractionation of HDL-cholesterol:

- Dextran sulfate (Mr 50,000) 40 gm/L, pH 7.0.
- $MgCl_2.6H_2O$, 2 M, pH 7.0.
- Reagent 'X' was prepared by mixing 1 volume of 40 gm/L dextran sulfate with 3 volumes of 2 M $MgCl_2.6H_2O$.

For HDL-cholesterol fractionation, 0.1 volume of reagent 'X' was added to 1 volume of HDL-cholesterol sample and mixed immediately. The samples were allowed to stand at room temperature for 15 min and then centrifuged at 5,000 rpm for 30 min at 4 °C. Aliquots of clear supernatant were used for HDL_3-cholesterol analysis. HDL_2-cholesterol were then calculated as the difference between HDL-cholesterol and HDL_3-cholesterol.

For the isolation of VLDL, 0.075 volume of 10% SDS solution was added to 1 volume of plasma. After mixing them well, the samples were incubated for 2 h at 37 °C. These were then centrifuged at 10,000 rpm for 10 minutes at room temperature. The VLDL pellicle floats at the top of the tube. The subnatant was carefully removed with a hypodermic syringe and discarded. The pellicle residue was dissolved in 1 volume of 1% SDS by warming at 37 °C for 15 minutes.

2.2.3.3 Determination of cholesterol

Total, free and esterified cholesterol were estimated in plasma and lipoproteins as described by Annino and Giese (1976). To glass centrifuge tubes, containing 4.8 ml of isopropanol, were added 0.2 ml of the test sample or 0.2 ml of the standard cholesterol (200 mg/100 ml isopropanol). The contents were mixed, allowed to stand for 5 min and centrifuged at 3,000 rpm for 10 min.

For the determination of cholesterol in plasma and subfractions, 1.0 ml of the supernatant was taken in glass test tubes and treated with 2.0 ml of ferric chloride reagent (70 mg of ferric chloride hexahydrate/100 ml of glacial acetic

acid), mixed immediately, followed by the addition of 2.0 ml of sulphuric acid with thorough mixing. For reagent blank, 1.0 ml of isopropanol was used instead of supernatant. After 5 min, the absorbance was read at 550 nm in a Beckman DU 640 spectrophotometer.

For the determination of free cholesterol and cholesterol ester, 1.0 ml of the above supernatant was treated with 2.0 ml of acetone and 1.0 ml of digitonin solution (1.0 g of digitonin dissolved in 60 ml of absolute ethanol, diluted to 100 ml with distilled water and mixed well). The tubes were allowed to stand in an ice bath for 30 min and then centrifuged for 10 min at 4,000 rpm. The supernatant was decanted completely. The precipitate was again washed with 3.0 ml of acetone and dissolved in 1.0 ml of isopropanol. At the same time, 1.0 ml isopropanol was taken in a clean tube to serve as blank. To all the tubes, 2.0 ml of ferric chloride reagent and 2.0 ml sulphuric acid were added and the contents were mixed well. After 5 min, the absorbance was read at 550 nm in a Beckman DU 640 spectrophotometer.

Calculations: Total, free and esterified cholesterol were calculated as follows :

Total cholesterol (mg/dl) = $\frac{\text{Absorbance of test sample}}{\text{Absorbance of standard}} \times 200$

Free cholesterol (mg/dl) = $\frac{\text{Absorbance of test sample}}{\text{Absorbance of standard}} \times 200$

Cholesterol ester (mg/dl) = Total cholesterol – Free cholesterol.

2.2.3.4 Determination of triglycerides

Triglycerides were determined by using enzymatic kit purchased from Pointe Scientific, Inc. (USA). The method uses a modified Trinder colour reaction to produce a fast, linear, end point reaction (Trinder, 1969).

Principle:

$$\text{Triglycerides} \xrightarrow{\text{Lipase}} \text{Glycerol + Fatty acids}$$

$$\text{Glycerol + ATP} \xrightarrow{\text{Glycerol kinase}} \text{G3P + ADP}$$

$$\text{G3P + } O_2 \xrightarrow{\text{Glycerophosphate oxidase}} \text{DAP + } H_2O_2$$

$$H_2O_2 + \text{4-AAP + TBHB} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine Dye + } 2H_2O$$

Triglycerides in the sample are hydrolyzed by lipase to glycerol and fatty acids. The glycerol is then phosphorylated by adenosine-5-triphosphate (ATP) to glycerol-3-phosphate (G3P) and adenosine-5-diphosphate (ADP) in a reaction catalyzed by glycerol kinase. Glycerol-3-phosphate is then converted to dihydroxyacetone phosphate (DAP) and hydrogen peroxide (H_2O_2) by glycerophosphate oxidase. The hydrogen peroxide then reacts with 4-amino-antipyrine (4-AAP) and 3-hydroxy-2,4,6-tribomobenzoic acid (TBHB) in a reaction catalyzed by peroxidase to yield a red coloured quinoneimine dye. The intensity of the colour produced after incubation is directly proportional to the concentration of triglycerides in the sample when measured at 540 nm.

Calculations: Triglycerides in a plasma sample were calculated as follows:

$$\textbf{Triglycerides (mg/dl)} = \frac{\text{OD Sample}}{\text{OD Standard}} \times n$$

[n = standard concentration (200 mg/dl)]

2.2.3.5 Determination of apoA-1 and apoB

ApoA-1 and apoB were determined in plasma samples by using kit from Spectrum (New Delhi). This test system permits the quantitative determination of apoA1 and apoB in plasma samples by turbidimetric analysis.

The reaction is based on the precipitation of antigen-antibody complexes provided from the monospecific antibody in determined quantity and the antigen, the apoA-1 or apoB present in the sample. The formation of this insoluble immuno-complex is accelerated in presence of polyethylene glycol buffer. The reaction is started by the addition of plasma containing the antigens. The optical density measured after incubation at 340 nm is proportional to the quantity of antigen present in the sample.

Calculations: ApoA-1 and apoB concentrations were determined by utilizing the optical density of each sample on a calibration curve constructed by using apoA-1 and apoB standards. The results were expressed as mg/dl.

2.2.3.6 Determination of lipid peroxidation in rat liver microsomes

Lipid peroxidation was induced by the method of Kamat *et al.* (1997) by using ascorbate-Fe^{2+} system. The incubation mixture (0.5 ml) contained i) 0.35 ml of Tris-HCl, pH 7.4 containing 1 mM KH_2PO_4; ii) 50 µl $FeSO_4$ in 0.1 mM HCl (final conc. 50 µM); iii) 50 µl microsomes; and iv) 50 µl ascorbic acid to give a final concentration of 0.4 mM. Incubation was carried out at 37 °C for 10 min in a shaker water bath. After incubation, the peroxidation product formed was measured as thiobarbituric acid reactive substances (TBARS) as described by Pushpendran *et al.* (1993). For estimating TBARS, the reactants were boiled for 15 min in a water bath with thiobarbituric acid reagent (0.5% 2-thiobarbituric acid/10% trichloro acetic acid/6 mM EDTA/0.63 M hydrochloric acid). After cooling, the precipitate formed was removed by centrifugation at 1,000 g for 10

min. The absorbance of the sample was determined at 532 nm against blank that contained all the reagents minus the microsomes.

Calculations: The values were calculated on the basis of molar extinction coefficient (MEC) of malondehyde (1.56×10^5 $M^{-1}cm^{-1}$).

$$\textbf{TBARS conc.} = \frac{\text{Absorbance}}{\text{MEC x Protein conc.}} = \text{nmole/min/mg protein}$$

Absorbance = Absorbance per min

2.2.3.7 Determination of LDL oxidation in plasma

2.2.3.7.1 Isolation of LDL from plasma

Plasma LDL were isolated by a precipitation method described by Wieland and Seidel (1983). The precipitation buffer consisted of 64 mM trisodium citrate adjusted to pH 5.05 with 5 N HCl, and contained 50,000 IU/L heparin. Before precipitation of LDL, plasma samples and precipitation reagents were allowed to equilibrate to room temperature. One milliliter of the sample was added to 7.0 ml of the heparin-citrate buffer. After mixing with a vortex mixer, the suspension was allowed to stand for 10 min at room temperature. The insoluble lipoproteins were then sedimented by centrifugation at 1,000 g for 10 min. The pellet was resuspended in 1.0 ml of 0.1 M Na-phosphate buffer, pH 7.4, containing 0.9% of NaCl.

2.2.3.7.2 Oxidation of LDL

LDL oxidation was induced by ascorbate-Fe^{2+}-system (Kamat *et al.*, 1997). The incubation mixture contained 0.35 ml of Tris-HCl, pH 7.4 containing 1 mM KH_2PO_4; 50 µM FeSO4 in 0.1 mM HCl; 50 µl of LDL and 0.4 mM of ascorbic acid. Incubation was carried out at 37 °C for 10 min in a shaker water bath. The

oxidation products formed were measured by the method of Buege and Aust (1978). The amount of conjugated dienes formed during LDL oxidation was assayed by measuring the absorbance of LDL at 234 nm against a buffer blank containing all the constituents except LDL.

Calculations: The amount of conjugated dienes formed were calculated on the basis of MEC of conjugated dienes ($2.52 \times 10^4\ M^{-1}cm^{-1}$).

$$\textbf{Conc. of conjugated dienes} = \frac{\text{O.D.}}{\text{MEC x Vol. of LDL}}$$

$$= \text{nmole/min/ml plasma}$$

O.D. = Change in optical density per min

2.2.3.8 Determination of HMG-CoA reductase activity in rat liver microsomes

2.2.3.8.1 Isolation of microsomes

Rat liver microsomes were isolated by the method of Edwards and Gould (1972) with minor modifications. Livers were homogenized at 4 °C in buffer A containing 0.04 M potassium phosphate, pH 7.2, 0.05 M KCl, 0.03 M potassium EDTA, 0.1 M sucrose (2.0 ml/g of liver) in a warring blender. The homogenate was centrifuged at 10,000 g for 20 min. The post-mitochondrial supernatant was centrifuged at 100,000 g for 1 h at 4 °C. The microsomal pellet was stored frozen at –20 °C.

2.2.3.8.2 Solubilization of HMG-CoA reductase

For optimal solubilization of the HMG-CoA reductase, method of Edwards *et al.* (1979) was used. The frozen microsomes were allowed to thaw either at room temperature or at 37 °C before addition of an equal volume of 50% glycerol in buffer B (buffer A plus 20 mM βME) preheated to 37 °C. The suspension was rehomogenized with 10 downward passes of a hand driven, all glass Potter-

Elvejhem homogenizer and then incubated at 37 °C for 60 min. The suspension was diluted three fold with buffer B preheated to 37 °C to a final glycerol concentration of 8.3%, rehomogenized with 10 downward passes of the glass homogenizer pestle and centrifuged at 100,000 g for 1 h at 25 °C. The supernatant containing solubilized HMG-CoA reductase was removed and stored at –20 °C for future use.

2.2.3.8.3 Assay of HMG-CoA reductase

HMG-CoA reductase activity in solubilized fractions (above) was assayed spectrophotometrically by the method of Kleinsek *et al.* (1977) with minor modifications. The activity of the solubilized HMG-CoA reductase was determined at 37 °C in a total volume of 0.25 ml using Beckman DU 640 spectrophotometer. Assay was carried out in buffer C containing 0.16 M potassium phosphate, pH 6.8, 0.2 M KCl, 0.004 M EDTA and 0.02 M βME. The reaction cell contained 100-200 μg protein, 0.2 mM NADPH and 0.1 mM HMG-CoA, whereas, control cell contained only 100-200 μg protein and 0.2 mM NADPH. Before the addition of HMG-CoA, the tubes were pre-incubated for 15 min. The reaction was stopped in cold water bath at 4 °C after incubation for 5 min at 37 °C. The change in absorbance from zero time was monitored at 340 nm against blank containing all the reagents except NADPH. The rate of oxidation of NADPH obtained in the linear range in control cell was subtracted from the rate obtained in the reaction cell.

One unit of enzyme activity is defined as the amount required to oxidize 2 nmole NADPH per min. Hence, one unit is equivalent to the synthesis of 1 nmole mevalonate per min.

Calculations:

Specific activity = $\frac{\text{O.D. x F}}{\text{Protein conc.}}$

= nmole of mevalonate/min/mg protein

O.D. = Change in optical density per min

F = Factor

2.2.3.9 Determination of HMG-CoA reductase protein mass by enzyme-linked immunosorbant assay (ELISA)

HMG-CoA reductase protein mass was determined by the method of Duckworth *et al.* (1991) with minor modifications. Assay was performed in 96 well maxisorp E plates (Nalgene NUNC). Plates were coated with 100 µl of microsomal enzyme (1-6 µg protein) diluted in 0.05 M carbonate-bicarbonate-coating buffer, pH 9.6. This was allowed to sit overnight at 4 °C. The protein solution was removed, and the wells were filled with 100 µl of 5% non-fat dry milk in Tris-buffered saline (TBS). This blocking solution was allowed to incubate for 3-4 h at room temperature. The blocking solution was removed and the plates were washed three times, for 2 min each, with 100 µl of Tween-Tris-buffered saline (TTBS) (20 mM Tris, pH 7.5, 500 mM NaCl, 0.05% Tween-20). The plates were then incubated with 50 µl of rat hepatic HMG-CoA reductase antibody for 2 h at 37 °C. The wells were again washed two times with TTBS. The secondary antibody consisted of 50 µl of goat anti-rabbit IgG conjugated with horseradish peroxidase (1:1000). This was allowed to incubate on the plates for 1-2 h. The wells were again washed two times with TTBS. Plates were developed by the addition of 100 µl of the 3,3',5,5'-tetramethyl benzidine peroxidase system. The colour reagent remained on the plates for 30 min, and the development was terminated by the addition of 100 µl of 1 M phosphoric acid. The absorbance was read at 450 nm on an automatic microplate reader.

Calculations: HMG-CoA reductase protein mass was calculated by utilizing the known amount of purified HMG-CoA reductase protein.

$$\textbf{HMG-CoA protein mass} = \frac{\text{Absorbance of Unknown}}{\text{Absorbance of Purified}} \times n$$

$$= \mu g/g \text{ of microsomal protein}$$

[n = Protein mass of purified HMG-CoA reducatse]

2.2.3.10 Protein estimation

The protein was determined by the method of Bradford (1976), using bovine serum albumin as standard. Aliquots of liver, brain, colon, pancreas and mammary gland homogenates and post-mitochondrial supernatants, microsomal suspension and solubilized HMG-CoA reductase fractions were first precipitated with 20% trichloroacetic acid. The protein pellets were dissolved in 1N NaOH and suitable aliquots were used for protein determination.

2.2.3.11 Statistical analysis

Statistical analysis of data was done by employing two tailed student t-test (Bennet and Franklin, 1967). P values less than 0.05 were considered significant.

Results

3.1 Percent Yield of Oil From Brans of Basmati and Saket-4 Cultivars and Percent Yield of Tocotrienol Rich Fraction (TRF) Isolated From These Oils and From Refined Edible Grade Rice Bran Oil

As seen in Table 1, the percent yield of crude rice bran oil (RBO) isolated from raw and parboiled of Basmati was 15% and 17%, respectively, whereas the percent yield of RBO from raw and parboiled Saket-4 was 12% and 14%, respectively. The yield of tocotrienol rich fraction (TRF) isolated from refined edible grade RBO was 4.3%, whereas yield of TRF from raw and parboiled Basmati and Saket-4 was approximately 6.9%. After purification of TRF on silica gel bed, the yield of purified TRF of commercial refined edible grade RBO was reduced from 4.3% to 2.8% due to removal of non-tocotrienol and non-tocopherol contaminants. The TRF and purified TRF, however, may be contaminated with minor amounts of oryzanol, polyphenols and/or steroids/terpenes. The average percent yield of purified TRF for raw and parboiled Basmati was 4.7%, whereas for Saket-4 it was 4.6%. These results demonstrate that yield of oil from parboiled cultivars of rice was slightly higher than the yield from the raw cultivars. The results also indicate that yield of TRF and purified TRF from crude RBO of Basmati and Saket-4 was approximately 68% higher than the TRF yield obtained from commercial refined edible grade RBO.

3.2 Quantification of Types and Content of Tocotrienols (T_3) and Tocopherols (T) by HPLC, Present in TRF and Purified TRF Isolated from Refined Edible Grade Rice Bran Oil and from Crude RBO of Two Cultivars of Rice

Table 2, represent the type and percent content of tocotrienols (T_3) and tocopherols (T) in TRF isolated from RBO, procured commercially and from Basmati and Saket-4, as determined by HPLC. The results were calculated from each peak area of the chromatogram of HPLC and are representative of the percent of the amount of sample loaded. The combined amount of T_3 in TRF and purified

TABLE 1

PERCENT YIELD OF OIL, TOCOTRIENOL RICH FRACTION (TRF) AND PURIFIED TRF ISOLATED FROM TWO CULTIVARS OF RICE, AND YIELD OF TRF AND PURIFIED TRF FROM REFINED EDIBLE GRADE RICE BRAN OIL (RBO)

Type	% Yield of Oil*	% Yield of TRF	% Yield of Purified TRF
Refined RBO	-	4.3 ± 0.27	2.8 ± 0.26
Raw Basmati	14.9 ± 0.21	7.2 ± 0.18	4.9 ± 0.12
Parboiled Basmati	17.3 ± 0.29	6.6 ± 0.15	4.5 ± 0.10
Raw Saket-4	12.4 ± 0.35	7.1 ± 0.15	4.8 ± 0.15
Parboiled Saket-4	13.6 ± 0.37	6.7 ± 0.11	4.3 ± 0.13

*Values are mean (g/100 g) ± SD of 3 separate determinations.

TABLE 2

QUANTIFICATION OF TYPES AND CONTENT OF TOCOTRIENOLS AND TOCOPHEROLS BY HPLC, PRESENT IN TRF AND PURIFIED TRF ISOLATED FROM REFINED EDIBLE GRADE RBO AND FROM CRUDE RBO OF TWO CULTIVARS OF RICE

Type	Refined RBO		Basmati		Saket-4	
	TRF	Purified TRF	Raw	Parboiled	Raw	Parboiled
Tocotrienols						
α-T_3	14.6	20.6	20.0	15.9	14.1	16.2
β-T_3	2.2	2.3	-	-	-	-
γ-T_3	6.2	9.7	5.0	9.7	24.9	19.3
δ-T_3	6.2	9.6	17.2	19.0	6.9	12.9
Total	29.2	42.2	42.2	44.6	45.9	48.1
Tocopherols						
α-T	14.7	20.6	20.1	21.7	24.2	19.4
β-T	10.6	16.1	10.1	9.3	29.9	22.1
γ-T	3.1	4.8	17.9	12.2	-	3.5
δ-T	10.7	16.4	9.7	12.2	-	6.6
Total	39.1	57.9	57.8	55.4	54.1	51.6

TRF from refined edible grade RBO was 29% and 42%, respectively, whereas that of T was 39% and 58%, respectively. Purified TRF from RBO of raw Basmati contained 42% T_3 and 59% T, whereas, purified TRF from RBO of parboiled Basmati contained 45% T_3 and 55% T. Purified TRF from raw Saket-4 contained 46% T_3 and 54% T, whereas, purified TRF from parboiled Saket-4 contained 48% T_3 and 52% T. The combined content of γ- and δ-T_3 in TRF from refined RBO was approximately 12%, whereas, γ- and δ-T_3 content in purified TRF from refined RBO was around 19%. The yield of γ- and δ-T_3 in purified TRF obtained from RBO of raw and parboiled Basmati was 22% and 29%, respectively. The total content of γ- and δ-T_3, in purified TRF isolated from RBO of raw and parboiled Saket-4 was 32% and 32%, respectively, (Table 2). These results demonstrate that TRF content as well as the individual type and quantity of T_3 and T vary from cultivar to cultivar of raw as well as parboiled rice.

3.3 Effect of Tocotrienol Rich Fraction and Purified Tocotrienol Rich Fraction (TRF) on Plasma Lipids, Plasma Lipoprotein Lipids, ApoB, ApoA-1, Activity and Protein Mass of HMG-CoA Reductase, Microsomal Lipid Peroxidation and Plasma LDL Oxidation in Normolipidemic Rats After Two Weeks of Treatment

In the experiments described below, the lipid lowering as well as antioxidant property of TRF and purified TRF was investigated in normolipidemic rats after two weeks of administration. The dose of purified TRF (**81.25 mg/kg/ day**) from refined RBO was adjusted to the dose calculated for TRF (**125 mg/kg/ day**) after removing the non-tocotrienol and non-tocopherol contaminants, which were approximately 35%.

3.3.1 Effect on plasma lipids

As shown in Table 3, administration of TRF (NLP-T1) and purified TRF (NLP-T2) to normolipidemic rats was associated with a significant decline in lipid

TABLE 3

EFFECT OF TRF AND PURIFIED TRF ON TRIGLYCERIDES, TOTAL, ESTERIFIED AND FREE CHOLESTEROL IN NORMOLIPIDEMIC RATS AFTER TWO WEEKS OF TREATMENT

Parameters	NLP-C	NLP-T1	NLP-T2
Triglycerides	47.9 ± 2.1*	33.2 ± 1.3 (-30.7%)‡	33.0 ± 1.6 (-31.1%)‡
Total cholesterol	73.4 ± 2.8	65.2 ± 2.1 (-11.2%)¶	64.4 ± 2.2 (-12.3%)¶
Esterified cholesterol	54.1 ± 0.9	47.1 ± 1.9 (-12.9%)‡	46.4 ± 1.4 (-14.5%)‡
Free cholesterol	19.3 ± 1.1	18.1± 0.9 (-6.2%)	18.0 ± 0.6 (-6.7%)

*Values are mean (mg/dl) ± SD from plasma of 5 individual rats in each group.
NLP-C, saline-fed control; NLP-T1 & NLP-T2, given 125 mg TRF and 81.25 mg purified TRF/kg/day, respectively, for 2 weeks.
Significantly different from NLP-C at ‡$p<0.001$, †$p<0.01$ & ¶$p<0.05$.

parameters. Plasma triglycerides showed a highly significant decline of approximately 31% whereas the decrease in total cholesterol was 12% in both the treated groups. Esterified cholesterol decreased by 13% and 15% in NLP-T1 and NLP-T2, respectively, whereas a decrease of approximately 6% in the free cholesterol was not significant (Table 3). These results demonstrate that TRF significantly reduces the plasma lipids in normolipidemic rats.

3.3.2 Impact on plasma lipoprotein lipids, apoB and apoA-1

As seen in Table 4 there was a significant decline in VLDL-C, LDL-C and HDL_3-C after two weeks of treatment of TRF and purified TRF. VLDL-C decreased by approximately 35% whereas the decline in LDL-C was 34-35% in NLP-T1 and NLP-T2. Although TRF and purified TRF did not affect HDL-C levels, HDL-C subfractions, HDL_3-C decreased by 11% and 15% in NLP-T1 and NLP-T2, respectively. In contrast, a significant increase of 31% and 38% in HDL_2-C was seen in NLPT-1 and NLP-T2, respectively, after two weeks of TRF and purified TRF administration. ApoB showed a considerable decrease and the decline was same in both the treated groups (19%) with no significant change in apoA-1 levels (Table 4). These results demonstrate that hypolipidemic impacts of TRF and purified TRF in normolipidemic rats were similar, apparently due to high dose of TRF (125 mg/kg/day) and purified TRF (81.25 mg/kg/day) used in these experiments.

3.3.3 Regulation of enzymatic activity and Protein Mass of solubilised Microsomal HMG-CoA reductase

In order to find out the mechanism of hypolipidemic action of TRF in normal rats, effect of TRF and purified TRF on hepatic HMG-CoA reductase activity as well as protein mass was investigated. The specific activity of HMG-CoA reductase was significantly reduced to 3.0 and 2.9 units/mg in NLP-T1 and NLP-T2, respectively, from 7.4 units/mg of saline-fed control rats (Table 5).

TABLE 4

IMPACT OF TRF AND PURIFIED TRF ON VLDL-C, LDL-C, HDL-C, HDL_2-C, HDL_3-C, ApoB AND ApoA-1 IN NORMOLIPIDEMIC RATS TREATED FOR TWO WEEKS

Parameters	NLP-C	NLP-T1	NLP-T2
VLDL-cholesterol	7.6 ± 0.5*	5.0 ± 0.4 (-34.2%)‡	4.9 ± 0.2 (-35.5%)‡
LDL-cholesterol	22.1 ± 1.4	14.6 ± 0.9 (-33.9%)‡	14.3 ± 0.6 (-35.3%)‡
HDL-cholesterol	43.7 ± 1.6	45.3 ± 1.2 (+3.7%)	45.4 ± 1.7 (+3.9%)
HDL_2-cholesterol	15.6 ± 0.8	20.4 ± 0.9 (+30.8%)‡	21.5 ± 0.8 (+37.8%)‡
HDL_3-cholesterol	28.1 ± 1.1	24.9 ± 1.1 (-11.4%)†	23.9 ± 1.1 (-14.9%)†
ApoB	14.0 ± 0.5	11.4 ± 0.7 (-18.6%)‡	11.4 ± 0.6 (-18.6%)‡
ApoA-1	92.9 ± 3.6	92.4 ± 2.3 (-0.5%)	92.5 ± 3.1 (-0.4%)

*Values are mean (mg/dl) ± SD from plasma of 5 individual rats in each group.
NLP-C, saline-fed control; NLP-T1 & NLP-T2, given 125 mg TRF and 81.25 mg purified TRF/kg/day, respectively, for 2 weeks.
Significantly different from NLP-C at ‡$p<0.001$ & †$p<0.01$.

TABLE 5

IN VIVO MODULATION OF ACTIVITY AND PROTEIN MASS OF HEPATIC HMG-CoA REDUCTASE IN NORMOLIPIDEMIC RATS TREATED WITH TRF AND PURIFIED TRF FOR TWO WEEKS

Group	HMG-CoA reductase	
	Specific activity*	Protein mass**
NLP-C	7.4 ± 1.0	212 ± 14
NLP-T1	3.0 ± 0.7 (-59.4%)‡	96 ± 8 (-54.7%)‡
NLP-T2	2.9 ± 0.5 (-60.8%)‡	91 ± 6 (-57.1%)‡

*Values are mean (nmoles of mevalonate formed/min/mg protein) ± SD from three separate assay in each group.

**Values are mean (μg/g of microsomal protein) ± SD from three separate assay in each group.

NLP-C, saline-fed control: NLP-T1 & NLP-T2, given 125 mg TRF and 81.25 mg purified TRF/kg/day, respectively, for 2 weeks.

Significantly different from NLP-C at ‡$p<0.001$.

Feeding of TRF (NLP-T1) and purified TRF (NLP-T2) caused a significant decline in the protein mass of HMG-CoA reductase by 55% and 57% respectively. These results demonstrate that TRF mediated decrease in lipid parameters of normolipidemic rat is due to decline in hepatic HMG-CoA reductase activity, which is due to reduction in its protein mass.

3.3.4 Impact on microsomal lipid peroxidation and plasma LDL oxidation

As shown in Table 6 TRF and purified TRF administration to normolipidemic rats significantly prevented microsomal lipid peroxidation and LDL oxidation measured *in vitro*. There was a significant decrease of 23% and 29% in thiobarbituric acid reactive substances (TBARS) formation, in NLP-T1 and in NLP-T2, respectively, when compared to untreated control rats. Whereas, formation of conjugated dienes, a reflection of plasma LDL oxidation was significantly decreased by approximately 42% in both the treated groups. These results demonstrate that TRF acts as a potent antioxidant in normolipidemic rats.

3.4 Effect of Purified Tocotrienol Rich Fraction on Plasma Lipids, Plasma Lipoprotein Lipids, ApoB, ApoA-1, Activity and Protein Mass of HMG-CoA Reductase, Microsomal Lipid Peroxidation and Plasma LDL Oxidation in Normolipidemic and Hyperlipidemic Rats Treated for Three Weeks

In the experiments described below the lipid lowering effect of purified TRF at dose of **65 mg/kg/day**, which is equivalent to dose of **100 mg TRF/kg/day**, in normolipidemic rats was investigated after three weeks of administration. The efficacy of purified TRF in preventing the increase in lipid parameters was also investigated by administering the same dose of 65 mg purified TRF/kg/day along with the atherogenic diet.

TABLE 6

EFFECT OF TRF AND PURIFIED TRF ON MICROSOMAL LIPID PEROXIDATION AND PLASMA LDL OXIDATION IN NORMOLIPIDEMIC RATS TREATED FOR TWO WEEKS

Group	TBARS*	Conjugated Diene**
NLP-C	1.88 ± 0.06	5.42 ± 0.22
NLP-T1	1.44 ± 0.10 (-23.4%)‡	3.12 ± 0.27 (-42.4%)‡
NLP-T2	1.34 ± 0.10 (-28.7%)‡	3.10 ± 0.19 (-42.8%)‡

*Values are mean (nmole/min/mg) ± SD of 4 separate assay in each group.
**Values are mean (nmole/min/ml) ± SD of 3 separate assay in each group.
NLP-C, saline-fed control; NLP-T1 & NLP-T2, given 125 mg TRF and 81.25 mg purified TRF/kg/day, respectively, for 2 weeks.
Significantly different from NLP-C at ‡$p<0.001$.

3.4.1 Impact on plasma lipids

When normolipidemic rats were treated with 65 mg purified TRF/kg/day for three weeks, a significant reduction in lipid parameters, lipoprotein lipids. HMG-CoA reductase activity and its mass was observed. As shown in Table 7, triglycerides reduced by 34%, whereas, the total, esterified and free cholesterol levels showed a significant decline of 15%, 17% and 10%, respectively, when compared to saline-fed control rats.

Feeding of atherogenic diet to rats (HLP-C) for three weeks, caused a substantial increase in all the lipid parameters. Triglycerides, total, esterified and free cholesterol increased from 48, 75, 54 and 20 mg/dl, respectively in NLP-C to 168, 185, 145 and 40 mg/dl in HLP-C, respectively. Administration of 65 mg purified TRF along with atherogenic diet to rats (HLP-T), significantly prevented the increase in lipid parameters when compared to HLP-C. The increase in triglycerides, total, esterified and free cholesterol was 191%, 81%, 89% and 61%, respectively in HLP-T, respectively, was significantly lower than an increase of 252%, 148%, 166% and 100%, respectively, in HLP-C (Table 7). These results demonstrate that purified TRF caused significant decline of plasma lipids in normolipidemic rats after three weeks of treatment, whereas, in hyperlipidemic rats purified TRF significantly prevented the increase in lipid parameters.

3.4.2 Effect on plasma lipoprotein lipids, apoB and apoA-1

As shown in Table 8 purified TRF administration to normolipidemic rats for three weeks caused a significant reduction in VLDL-C, LDL-C and HDL_3-C by 47%, 35% and 18%, respectively, with no significant effect on HDL-C. HDL_2-C was significantly increased by 35%. ApoB level was significantly decreased by 21% when compared to saline-fed control rats, whereas apoA-1 level remained unaffected.

TABLE 7

EFFECT OF PURIFIED TRF ON TRIGLYCERIDES, TOTAL, ESTERIFIED AND FREE CHOLESTEROL IN NORMOLIPIDEMIC AND HYPERLIPIDEMIC RATS AFTER THREE WEEKS OF TREATMENT

Parameters	NLP-C	NLP-T	HLP-C	HLP-T
Triglycerides	47.8 ± 2.1*	31.7 ± 1.0 (-33.7%)‡	168.3 ± 5.2 (+252.1%)‡	139.0 ± 4.4 (+190.8%)‡
Total cholesterol	74.5 ± 3.1	63.0 ± 1.9 (-15.4%)‡	185.0 ± 6.5 (+148.3%)‡	135.2 ± 3.7 (+81.5%)‡
Esterified cholesterol	54.4 ± 1.7	44.9 ± 1.9 (-17.5%)‡	144.7 ± 5.7 (+166.0%)‡	102.8 ± 3.3 (+89.0%)‡
Free cholesterol	20.1 ± 0.7	18.1 ± 0.7 (-9.9%)†	40.2 ± 1.6 (+100.0%)‡	32.3 ± 0.6 (+60.7%)‡

*Values are mean (mg/dl) ± SD from plasma of 3 individual rats in each group.
NLP-C, saline-fed control; NLP-T, given 65 mg purified TRF/kg/day for 3 weeks; HLP-C, hyperlipidemic control; HLP-T, given 65 mg purified TRF/kg/day alongwith the atherogenic diet for 3 weeks.
Significantly different from NLP-C at ‡$p<0.001$ & †$p<0.01$.

TABLE 8

IMPACT OF PURIFIED TRF ON VLDL-C, LDL-C, HDL-C, HDL_2-C, HDL_3-C, ApoB AND ApoA-1 IN NORMOLIPIDEMIC AND HYPERLIPIDEMIC RATS TREATED FOR THREE WEEKS

Parameters	NLP-C	NLP-T	HLP-C	HLP-T
VLDL-cholesterol	8.0 ± 0.4*	4.2 ± 0.3 (-47.5%)‡	16.0 ± 0.4 (+100.0%)‡	11.4 ± 0.4 (+42.5%)‡
LDL-cholesterol	23.0 ± 1.2	15.0 ± 0.8 (-34.8%)‡	104.0 ± 4.3 (+352.2%)‡	68.5 ± 1.2 (+197.8%)‡
HDL-cholesterol	43.5 ± 1.5	43.8 ± 1.1 (+0.7%)	64.9 ± 2.4 (+49.2%)‡	55.0 ± 1.1 (+26.4%)‡
HDL_2-cholesterol	15.3 ± 0.7	20.7 ± 0.5 (+35.3%)‡	23.0 ± 0.9 (+50.3%)‡	23.0 ± 0.9 (+50.3%)‡
HDL_3-cholesterol	28.2 ± 1.1	23.1 ± 0.8 (-18.1%)‡	41.9 ± 1.2 (+48.6%)‡	32.0 ± 1.1 (+13.5%)†
ApoB	14.6 ± 0.6	11.5 ± 0.6 (-21.2%)‡	62.2 ± 2.0 (+326.0%)‡	42.9 ± 1.4 (+193.8%)‡
ApoA-1	92.5 ± 3.4	92.4 ± 2.6 (-0.1%)	107.9 ± 3.9 (+16.6%)‡	101.6 ± 3.1 (+9.8%)†

*Values are mean (mg/dl) ± SD from plasma of 3 rats in each group.
NLP-C, saline-fed control; NLP-T, given 65 mg purified TRF/kg/day for 3 weeks; HLP-C, hyperlipidemic control; HLP-T, given 65 mg purified TRF/kg/day alongwith the atherogenic diet for 3 weeks.
Significantly different from NLP-C at ‡$p<0.001$ & †$p<0.01$.

Plasma lipoprotein lipids, apoB and apoA-1 levels were significantly increased on feeding atherogenic diet for three weeks. This increase was significantly arrested by administration of 65 mg purified TRF along with atherogenic diet. The increase in VLDL-C was 100% in HLP-C whereas an increase of only 43% was observed in purified TRF treated (HLP-T) group (Table 8). Similarly, purified TRF prevented the increase of LDL-C, HDL-C and HDL_3-C substantially. The increase in LDL-C, HDL-C and HDL_3-C levels was 198%, 26% and 13%, respectively, in HLP-T, compared to an increase of 352%, 49% and 49%, respectively, in HLP-C rats, whereas no effect was seen on HDL_2-C level. ApoB and apoA-1 levels increased by 326% and 17%, respectively, in HLP-C whereas an increase of 194% and 10%, respectively, was observed in HLP-T, after three weeks of TRF feeding (Table 8). These results demonstrate that purified TRF caused the significant decline in VLDL-C, LDL-C and apoB levels with no significant affect on HDL-C and apoA1 levels in normolipidemic rats after 3 weeks of TRF treatment. Feeding of purified TRF along with atherogenic diet for 3 weeks significantly prevented the increase in lipoprotein lipids including apoB.

3.4.3 Regulation of enzymatic activity and Protein Mass of solubilized Microsomal HMG-CoA reductase

As depicted in Table 9, HMG-CoA reductase activity and protein mass was significantly decreased by 54% and 49%, respectively, in normolipidemic rats treated with TRF (NLP-T) for 3 weeks in comparison to saline-fed control rats. Feeding of atherogenic diet caused a suppression of HMG-CoA reductase activity and protein mass. The specific activity of HMG-CoA reductase in HLP-C was 2.1 units/mg as compared to 7.4 units/mg in NLP-C, indicating a suppression of 72%. Similarly, a reduction of 49% in protein mass was observed in HLP-C. Purified TRF feeding along with atherogenic diet caused a further decrease in the

TABLE 9

REGULATION OF ACTIVITY AND PROTEIN MASS OF HEPATIC HMG-CoA REDUCTASE IN NORMOLIPIDEMIC AND HYPERLIPIDEMIC RATS TREATED WITH PURIFIED TRF FOR THREE WEEKS

Group	HMG-CoA reductase	
	Specific activity*	Protein mass**
NLP-C	7.4 ± 0.8	247 ± 3
NLP-T	3.4 ± 0.5 (-54.0%)‡	126 ± 4 (-49.0%)‡
HLP-C	2.1 ± 0.4 (-71.6%)‡	125 ± 3 (-49.4%)‡
HLP-T	1.8 ± 0.2 (-75.7%)‡	96 ± 4 (-61.1%)‡

*Values are mean (nmoles of mevalonate formed/min/mg protein) ± SD from three separate assay in each group.

**Values are mean (μg/g of microsomal protein) ± SD from three separate assay in each group.

NLP-C, saline-fed control; NLP-T, given 65 mg purified TRF/kg/day for 3 weeks; HLP-C, hyperlipidemic control; HLP-T, given 65 mg purified TRF/kg/day alongwith the atherogenic diet for 3 weeks.

Significantly different from NLP-C at ‡$p<0.001$.

enzymatic activity (76%) and protein mass (61%) of HMG-CoA reductase (Table 9). These results demonstrate that decrease in lipid parameters of normolipidemic rat is due to decline in hepatic HMG-CoA reductase activity, which is due to reduction in its protein mass. Feeding of atherogenic diet caused a significant decrease in HMG-CoA reductase activity and protein mass, which was further declined when purified TRF was administered along with atherogenic diet.

3.4.4 Impact on microsomal lipid peroxidation and LDL oxidation

As shown in Table 10, a significant decrease of 34% in TBARS formation was observed in normolipidemic rats treated with purified TRF. The formation of conjugated dienes was significantly decreased by 44% after three weeks of purified TRF administration to normolipidemic rats. Feeding of atherogenic diet caused an increase in TBARS and conjugated dienes formation by 96% and 79%, respectively, as compared to values obtained from NLP-C rats. Administration of purified TRF along with atherogenic diet (HLP-T) for 3 weeks significantly blocked the increase in TBARS and conjugated dienes formation by 62% and 66%, respectively (Table 10). These results demonstrate that purified TRF significantly prevented the lipid peroxidation and oxidation of LDL in normo-lipidemic rats. The results also demonstrate that the induction of hyperlipidemia in rats is associated with a significant increase in the microsomal lipid peroxidation as well as plasma LDL oxidation, which is significantly prevented by the administration of purified TRF.

TABLE 10

IMPACT OF PURIFIED TRF ON MICROSOMAL LIPID PEROXIDATION AND PLASMA LDL OXIDATION IN NORMOLIPIDEMIC AND HYPERLIPIDEMIC RATS TREATED FOR 3 WEEKS

Group	TBARS*	Conjugated Diene**
NLP-C	1.94 ± 0.08	5.50 ± 0.26
NLP-T	1.27 ± 0.10 (-34.5%)‡	3.10 ± 0.29 (-43.6%)‡
HLP-C	3.81 ± 0.13	9.85 ± 0.28
HLP-T	1.46 ± 0.14 (-61.7%)‡	3.39 ± 0.23 (-65.6%)‡

*Values are mean (nmole/min/mg) ± SD of 4 separate assay in each group.
**Values are mean (nmole/min/ml) ± SD of 3 separate assay in each group.
NLP-C, saline-fed control; NLP-T, given 65 mg purified TRF/kg/day for 3 weeks; HLP-C, hyperlipidemic control; HLP-T, given 65 mg purified TRF/kg/day alongwith the atherogenic diet for 3 weeks.
Significantly different from respective controls, NLP-C and HLP-C at ‡$p<0.001$.

3.5 Effect of Purified Tocotrienol Rich Fraction on Plasma Lipids, Plasma Lipoprotein Lipids, ApoB, ApoA-1, Activity and Protein Mass of HMG-CoA Reductase, Microsomal Lipid Peroxidation and Plasma LDL Oxidation in Hyperlipidemic Rats Treated for 5 Days After Withdrawal of Atherogenic Diet

As expected, the efficacy in terms of lipid lowering property of TRF at 125 mg/kg/day and purified TRF at an equivalent dose of 81.25 mg/kg/day was quite similar in normolipidemic rats after two weeks of administration. In order to find out the impact of purified TRF on cholesterol dynamics in experimentally induced hyperlipidemic rats, a dose of **16.25 and 32.50 mg purified TRF/kg/day**, equivalent to **25 and 50 mg TRF/kg/day**, respectively, was administered for 5 days.

3.5.1 Effect on plasma lipids

As seen in Table 11, feeding of an atherogenic diet for three weeks caused an increase in plasma lipids and lipoproteins. However, 5 days after the withdrawal of atherogenic diet, a decrease in these parameters was observed in HLP-UC group. The decrease in these parameters was more marked when 16.25 (HLP-T1) and 32.50 mg (HLP-T2) purified TRF was given for 5 days. There was significant decline in triglycerides and total cholesterol levels by 11% and 18%, respectively, in both the treated groups, when compared to HLP-UC1. Esterified cholesterol decreased by 20% (HLP-T1) and 19% (HLP-T2), whereas free cholesterol decreased by approximately 13% in both the treated groups (Table 11). These results demonstrate a significant TRF-mediated decline in lipid parameters after 5 days of administration to hyperlipidemic rats.

3.5.2 Effect on plasma lipoprotein lipids, apoB and apoA-1

As shown in Table 12, there was an insignificant decline of 4% in VLDL-C in HLP-T1, but VLDL-C decreased (10%) significantly in HLP-T2, whereas a significant decline of 21% was observed in LDL-C in both the TRF

TABLE 11

EFFECT OF PURIFIED TRF ON TRIGLYCERIDES, TOTAL, ESTERIFIED AND FREE CHOLESTEROL IN HYPERLIPIDEMIC RATS TREATED FOR 5 DAYS

Parameters	HLP-C	HLP-UC1	HLP-T1	HLP-T2
Triglycerides	168.3 ± 5.2*	151.8 ± 6.1	135.6 ± 4.5 (-10.7%)†	135.7 ± 6.1 (-10.6%)†
Total cholesterol	185.0 ± 6.5	159.6 ± 6.2	130.8 ± 5.2 (-18.0%)‡	130.7 ± 4.6 (-18.1%)‡
Esterified cholesterol	144.7 ± 5.7	122.2 ± 4.9	98.1 ± 3.5 (-19.7%)‡	98.4 ± 3.4 (-19.5%)‡
Free cholesterol	40.2 ± 1.6	37.4 ± 1.3	32.7 ± 1.7 (-12.6%)†	32.3 ± 1.4 (-13.6%)‡

*Values are mean (mg/dl) ± SD from plasma of 3 individual rats in each group.
HLP-C, hyperlipidemic control; HLP-UC1, saline-fed control; HLP-T1 & HLP-T2, given 16.25 mg and 32.50 mg purified TRF/kg/day, respectively, for 5 days.
Significantly different from HLP-UC1 at ‡$p<0.001$ & †$p<0.01$.

TABLE 12

IMPACT OF PURIFIED TRF ON VLDL-C, LDL-C, HDL-C, HDL_2-C, HDL_3-C, ApoB AND ApoA-1 IN HYPERLIPIDEMIC RATS AFTER 5 DAYS OF TREATMENT

Parameters	HLP-C	HLP-UC1	HLP-T1	HLP-T2
VLDL-cholesterol	16.0 ± 0.4*	13.7 ± 0.4	13.1 ± 0.6 (-4.4%)	12.3 ± 0.9 (-10.2%)¶
LDL-cholesterol	104.0 ± 4.3	85.6 ± 2.2	67.6 ± 3.0 (-21.0%)‡	67.5 ± 2.1 (21.1%)‡
HDL-cholesterol	64.9 ± 2.4	60.0 ± 1.2	50.0 ± 1.9 (-16.7%)‡	50.7 ± 1.8 (-15.5%)‡
HDL_2-cholesterol	23.0 ± 0.9	24.7 ± 0.7	18.2 ± 0.7 (-26.3%)‡	19.3 ± 1.0 (-21.9%)‡
HDL_3-cholesterol	41.9 ± 1.2	35.3 ± 0.8	31.8 ± 1.5 (-9.9%)†	31.4 ± 1.7 (-11.0%)†
ApoB	62.2 ± 2.0	53.4 ± 2.0	42.0 ± 1.1 (-21.3%)‡	42.6 ± 2.1 (-20.2%)‡
ApoA-1	107.9 ± 3.9	104.6 ± 4.1	100.2 ± 2.9 (-4.2%)	100.7 ± 2.1 (-3.7%)

*Values are mean (mg/dl) ± SD from plasma of 3 individual rats in each group.
HLP-C, hyperlipidemic control; HLP-UC1, saline-fed control; HLP-T1 & HLP-T2, given 16.25 mg and 32.50 mg purified TRF/kg/day, respectively, for 5 days.
Significantly different from HLP-UC1 at ‡$p<0.001$, †$p<0.01$ & ¶$p<0.05$.

treated groups. The levels of HDL-C, HDL_2-C and HDL_3-C were decreased by 17% , 26% and 10% in HLP-T1, respectively, and the decrease in HLP-T2 was 15%, 22% and 11% respectively, as compared to HLP-UC1 after 5 days of treatment. ApoB levels were significantly decreased by 20% to 21% in both the treated groups, with no significant change in apoA-1 levels (Table 12). These results demonstrate that feeding of atherogenic diet causes a significant increase of VLDL-C, LDL-C and apoB levels. As can be seen, administration of purified TRF to hyperlipidemic rats for 5 days substantially decrease the above lipid parameters including apoB.

3.5.3 Modulation of HMG-CoA reductase activity and its protein mass

HMG-CoA reductase activity and its protein mass was decreased by 47% and 31%, respectively, in HLP-T1 as compared to HPL-UC1 after 5 days of purified TRF treatment as shown in Table 13. The level of reduction in HMG-CoA reductase activity and protein mass in HLP-T2 was similar to HLP-T1. These results indicate that feeding of exogenous cholesterol or purified TRF to rats decrease the synthesis of endogenous cholesterol via decrease in HMG-CoA reductase activity which in turn is mediated by the decrease in its protein mass. However, withdrawal of atherogenic diet relieves this inhibition which is evident from the increase in HMG-CoA reductase activity as well as its protein mass.

3.5.4 Impact on microsomal lipid peroxidation and LDL oxidation

As shown in Table 14, formation of TBARS in microsomes was increased from 1.94 units/mg in NLP-C to 3.81 units/mg in HLP-C, whereas LDL oxidation in plasma increased from 5.50 units/ml in NLP-C to 9.85 units/ml in HLP-C, after feeding atherogenic diet for three weeks. These values were reduced to 3.09 units/mg and 8.26 units/ml in HLP-UC1 after 5 days of the withdrawal of atherogenic diet, but the decrease was enhanced significantly in purified TRF treated hyperlipidemic rats for 5 days. Lipid peroxidation in microsomes decreased

TABLE 13

IN VIVO MODULATION OF ACTIVITY AND PROTEIN MASS OF HEPATIC HMG-CoA REDUCTASE IN HYPERLIPIDEMIC RATS TREATED WITH PURIFIED TRF FOR 5 DAYS

Group	HMG-CoA reductase	
	Specific activity*	Protein mass**
HLP-C	2.1 ± 0.4	125 ± 3
HLP-UC1	3.8 ± 0.3	142 ± 5
HLP-T1	2.0 ± 0.2 (-47.4%)‡	98 ± 3 (-31.0%)‡
HLP-T2	2.0 ± 0.3 (-47.4%)‡	95 ± 4 (-33.1%)‡

*Values are mean (nmoles of mevalonate formed/min/mg protein) ± SD from three separate assay in each group.

**Values are mean (μg/g of microsomal protein) ± SD from three separate assay in each group.

HLP-C, hyperlipidemic control; HLP-UC1, saline-fed control; HLP-T1 & HLP-T2, given 16.25 mg and 32.50 mg purified TRF/kg/day, respectively, for 5 days.

Significantly different from HLP-UC1 at ‡$p<0.001$.

TABLE 14

IMPACT OF PURIFIED TRF ON MICROSOMAL LIPID PEROXIDATION AND PLASMA LDL OXIDATION IN HYPERLIPIDEMIC RATS AFTER 5 DAYS OF TREATMENT

Group	TBARS*	Conjugated Diene**
NLP-C	1.94 ± 0.08	5.50 ± 0.26
HLP-C	3.81 ± 0.13	9.85 ± 0.28
HLP-UC1	3.09 ± 0.12	8.26 ± 0.26
HLP-T1	1.61 ± 0.11 (-38.5%)‡	4.55 ± 0.24 (-42.8%)‡
HLP-T2	1.55 ± 0.11 (-41.2%)‡	4.40 ± 0.18 (-44.4%)‡

*Values are mean (nmole/min/mg) ± SD of 4 separate assay in each group.
**Values are mean (nmole/min/ml) ± SD of 3 separate assay in each group.
HLP-C, hyperlipidemic control; HLP-UC1, saline-fed control; HLP-T1 & HLP-T2, given 16.25 mg and 32.50 mg purified TRF/kg/day, respectively, for 5 days.
Significantly different from HLP-UC2 at ‡$p<0.001$.

by 39%, and 41% in HLP-T1 and HLP-T2, respectively, whereas formation of conjugated dienes in plasma was reduced by approximately 43%, in both the treated groups. (Table 14). These results demonstrate that the induction of hyperlipidemia in rats is associated with a significant increase in the microsomal lipid peroxidation as well as plasma LDL oxidation, which is significantly prevented by the administration of purified TRF.

3.6 Effect of Purified Tocotrienol Rich Fraction on Plasma Lipids, Plasma Lipoprotein Lipids, ApoB, ApoA-1, Activity and Protein Mass of HMG-CoA Reductase, Microsomal Lipid Peroxidation and Plasma LDL Oxidation in Hyperlipidemic Rats Treated for 7 Days After Withdrawal of Atherogenic Diet

In experiments described below, the impact of purified TRF on cholesterol dynamics in experimentally induced hyperlipidemic rats was investigated. A dose of **16.25 and 32.50 mg purified TRF/kg/day**, equivalent to **25 and 50 mg TRF/kg/day**, was administered for 7 days.

3.6.1 Effect on plasma lipids

As shown in Table 15, a highly significant decrease of 34% in triglycerides concentration was observed in hyperlipidemic rats treated with 16.25 mg (HLP-T3) and 32.50 mg (HLP-T4) of purified for 7 days when compared to HPL-UC2, the saline-fed untreated control rats. Similarly, total, esterified and free cholesterol levels showed a significant reduction of 36%, 38% and 33%, respectively, in HLP-T3, whereas, the reduction in HLP-T4 was approximately same as in HLP-T3 (Table 15). These results demonstrate that reduction in lipid parameters was achieved at a faster rate when treated with TRF as compared to untreated hyperlipidemic control group.

TABLE 15

EFFECT OF PURIFIED TRF ON TRIGLYCERIDES, TOTAL, ESTERIFIED AND FREE CHOLESTEROL IN HYPERLIPIDEMIC RATS TREATED FOR 7 DAYS

Parameters	HLP-C	HLP-UC2	HLP-T3	HLP-T4
Triglycerides	168.3 ± 5.2*	134.0 ± 6.0	88.5 ± 3.3 (-34.0%)‡	88.6 ± 3.1 (-33.8%)‡
Total cholesterol	185.0 ± 6.5	129.7 ± 6.3	82.4 ± 3.6 (-36.5%)‡	83.7 ± 2.2 (-35.5%)‡
Esterified cholesterol	144.7 ± 5.7	97.8 ± 5.2	60.9 ± 2.6 (-37.7%)‡	62.0 ± 2.1 (-36.6%)‡
Free cholesterol	40.2 ± 1.6	31.9 ± 1.1	21.5 ± 1.1 (-32.6%)‡	21.7 ± 1.4 (-32.0%)‡

*Values are mean (mg/dl) ± SD from plasma of 3 individual rats in each group.
HLP-C, hyperlipidemic control; HLP-UC2, saline-fed control; HLP-T3 & HLP-T4, given 16.25 mg and 32.50 mg purified TRF/kg/day, respectively, for 7 days.
Significantly different from HLP-UC2 at ‡$p<0.001$.

3.6.2 Effect on plasma lipoprotein lipids, apoB and apoA-1

VLDL-C decreased significantly by approximately 23% in HLP-T3 and HLP-T4 when compared to HLP-UC2 after 7 days of treatment (Table 16). The decline in LDL-C was approximately 58% in HLP-T3 and in HLP-T4 rats. Reduction in HDL-C and HDL_2-C was approximately same in both the treated groups, i.e. 9.0% and 16.0%, respectively, with no significant change in HDL_3-C concentration. ApoB levels showed a significant decline of 53% in both the treated groups as compared to HLP-UC2 group, whereas apoA-I levels were not affected after 7 days of purified TRF treatment (Table 16). These results demonstrate that decline in total cholesterol was due to a significant decrease in VLDL-C and LDL-C. In addition. a reduction in apoB levels, which is associated with LDL was also seen.

3.6.3 Regulation of hepatic HMG-CoA reductase activity and protein mass

As seen in Table 17, after 7 days of purified TRF treatment, HMG-CoA reductase activity and protein mass was decreased by 60% and 52%, respectively, in HLP-T3 as compared to HLP-UC2 group. Similarly a decrease of 59% and 53% in enzymatic activity and protein mass of HMG-CoA reductase was observed in HLP-T4. (Table 17). These results demonstrate that reduction in HMG-CoA reductase activity and protein mass at the two doses of purified TRF is almost similar.

3.6.4 Impact on microsomal lipid peroxidation and LDL oxidation

As shown in Table 18, formation of TBARS in microsomes was increased from 1.94 units/mg in NLP-C to 3.81 units/mg in HLP-C, whereas LDL oxidation in plasma increased from 5.50 units/ml in NLP-C to 9.85 units/ml in HLP-C, after feeding atherogenic diet for three weeks. These values were reduced to 2.21 units/mg and 6.40 units/ml in HLP-UC1 after 7 days of the withdrawal of atherogenic diet, but the decrease was enhanced significantly in purified TRF

TABLE 16

IMPACT OF PURIFIED TRF ON VLDL-C, LDL-C, HDL-C, HDL_2-C, HDL_3-C, ApoB AND ApoA-1 IN HYPERLIPIDEMIC RATS AFTER 7 DAYS OF TREATMENT

Parameters	HLP-C	HLP-UC2	HLP-T3	HLP-T4
VLDL-cholesterol	16.0 ± 0.4*	11.4 ± 0.9	8.9 ± 0.8 (-21.9%)‡	8.7 ± 0.5 (-23.7%)‡
LDL-cholesterol	104.0 ± 4.3	66.8 ± 2.2	27.1 ± 2.4 (-59.4%)‡	28.6 ± 1.4 (-57.2%)‡
HDL-cholesterol	64.9 ± 2.4	50.9 ± 1.8	46.3 ± 1.2 (-9.0%)†	46.4 ± 1.4 (-8.8%)†
HDL_2-cholesterol	23.0 ± 0.9	20.0 ± 0.8	16.8 ± 1.01 (-16.0%)†	16.9 ± 1.0 (-15.5%)†
HDL_3-cholesterol	41.9 ± 1.2	30.9 ± 1.2	29.5 ± 3.6 (-4.5%)	29.5 ± 2.1 (-4.5%)
ApoB	62.2 ± 2.0	44.4 ± 1.4	20.9 ± 0.7 (-52.9%)‡	20.5 ± 1.5 (-53.8%)‡
ApoA-1	107.9 ± 3.9	100.7 ± 4.7	94.5 ± 4.1 (-6.1%)	95.2 ± 3.8 (-5.5%)

*Values are mean (mg/dl) ± SD from plasma of 3 individual rats in each group.
HLP-C, hyperlipidemic control; HLP-UC2, saline-fed control; HLP-T3 & HLP-T4, given 16.25 mg and 32.50 mg purified TRF/kg/day, respectively, for 7 days.
Significantly different from HLP-UC2 at ‡$p<0.001$ & †$p<0.01$.

TABLE 17

REGULATION OF ACTIVITY AND PROTEIN MASS OF HEPATIC HMG-CoA REDUCTASE IN HYPERLIPIDEMIC RATS TREATED WITH PURIFIED TRF FOR 7 DAYS

Group	HMG-CoA reductase	
	Specific activity*	Protein mass**
HLP-C	2.1 ± 0.4	125 ± 3
HLP-UC2	6.5 ± 0.5	219 ± 5
HLP-T3	2.6 ± 0.3 (-60.0%)‡	105 ± 5 (-52.0%)‡
HLP-T4	2.7 ± 0.3 (-58.5%)‡	104 ± 3 (-52.5%)‡

*Values are mean (nmoles of mevalonate formed/min/mg protein) ± SD from three separate assay in each group.

**Values are mean (μg/g of microsomal protein) ± SD from three separate assay in each group.

HLP-C, hyperlipidemic control; HLP-UC2, saline-fed control; HLP-T3 & HLP-T4, given 16.25 mg and 32.50 mg purified TRF/kg/day, respectively, for 7 days.

Significantly different from HLP-UC2 at ‡$p<0.001$.

TABLE 18

IMPACT OF PURIFIED TRF ON MICROSOMAL LIPID PEROXIDATION AND PLASMA LDL OXIDATION IN HYPERLIPIDEMIC RATS AFTER 7 DAYS OF TREATMENT

Group	TBARS*	Conjugated Diene**
NLP-C	1.94 ± 0.08	5.50 ± 0.26
HLP-C	3.81 ± 0.13	9.85 ± 0.28
HLP-UC2	2.21 ± 0.16	6.40 ± 0.31
HLP-T3	1.36 ± 0.10 (-38.5%)‡	3.66 ± 0.19 (-42.8%)‡
HLP-T4	1.30 ± 0.10 (-41.2%)‡	3.56 ± 0.22 (-44.4%)‡

*Values are mean (nmole/min/mg) ± SD of 4 separate assay in each group.
**Values are mean (nmole/min/ml) ± SD of 3 separate assay in each group.
HLP-C, hyperlipidemic control; HLP-UC2, saline-fed control; HLP-T3 & HLP-T4, given 16.25 mg and 32.50 mg purified TRF/kg/day, respectively, for 7 days.
Significantly different from HLP-UC2 at ‡$p<0.001$.

treated hyperlipidemic rats for 7 days. Lipid peroxidation in microsomes decreased by 39%, and 41% in HLP-T3 and HLP-T4, respectively, whereas formation of conjugated dienes in plasma was reduced by approximately 43%, in both the treated groups. (Table 18). These results demonstrate that the induction of hyperlipidemia in rats is associated with a significant increase in the microsomal lipid peroxidation as well as plasma LDL oxidation, which is significantly prevented by the administration of purified TRF.

3.7 Dose-Dependent Impact of Tocotrienol Rich Fraction on Plasma Lipids, Plasma Lipoproteins, ApoB, ApoA-1, Activity and Protein Mass of HMG-CoA Reductase and Microsomal Lipid Peroxidation and Plasma LDL Oxidation in Normolipidemic Rats After 3 Weeks of Treatment

It is evident from the above combined results that efficacy in terms of lipid lowering property of TRF and purified TRF in normolipideic rats at the doses of 81.25 mg or 65 mg for 2 and 3 weeks respectively was quite similar. Therefore, in order to find out the optimal dose of TRF in terms of its maximal hypolipidemic activity, **4, 8 and 12 mg TRF/kg/day** was administered to normolipidemic as well as hyperlipidemic rats.

3.7.1 Impact on plasma lipids

Data presented in Table 19, demonstrate that administration of 4, 8 and 12 mg TRF/kg/day for 3 weeks resulted in the decrease of lipid parameters. At 4 mg TRF the decrease in triglyceride was 25% whereas a decrease of 33% and 35% was observed at 8 and 12 mg TRF/kg/day. The decline in total cholesterol content was not significant at 4 mg TRF. However, a significant decline of 8% and 10% was observed at 8 and 12 mg TRF. Esterified cholesterol was insignificantly reduced by 7% at 4 and 8 mg TRF, but significantly decreased by 10% at 12 mg TRF. Three doses of TRF did not influence the free cholesterol level after 3 weeks of treatment. (Table 19). These results demonstrate that there is

TABLE 19

DOSE-DEPENDENT EFFECT OF TRF ON TRIGLYCERIDES, TOTAL, ESTERIFIED AND FREE CHOLESTEROL IN NORMOLIPIDEMIC RATS AFTER 3 WEEKS TREATMENT

Parameters	NLP-C	NLP-T1	NLP-T2	NLP-T3
Triglycerides	51.2 ± 2.79*	38.6 ± 1.55 (-24.6%)‡	34.1 ± 1.20 (-33.4%)‡	33.1 ± 1.80 (-35.3%)‡
Total cholesterol	72.8 ± 1.76	69.3 ± 1.48 (-4.8%)	66.6 ± 1.60 (-8.5%)¶	65.8 ± 1.90 (-9.6%)†
Esterified cholesterol	53.0 ± 2.29	49.1 ± 1.65 (-7.3%)	49.3 ± 0.83 (-7.0%)	47.8 ± 0.95 (-9.8%)¶
Free cholesterol	19.9 ± 1.20	20.2 ± 0.36 (+1.5%)	17.3 ± 1.12 (-13.1%)	18.0 ± 1.10 (-9.5%)

*Values are mean (mg/dl) ± SD from plasma of 3 individual rats in each group.
NLP-C, saline-fed control; NLP-T1, NLP-T2 & NLP-T3, given 4 mg, 8 mg and 12 mg TRF/kg/day, respectively, for 3 weeks.
Significantly different from NLP-C at ‡$p<0.001$, †$p<0.01$ & ¶$p<0.05$.

a differential decrease in lipid parameters at 3 doses of TRF with a maximum affect observed at 8 mg TRF/kg/day.

3.7.2 Effect on plasma lipoprotein lipids, apoB and apoA-1

As shown in Table 20, VLDL-C level was insignificantly reduced by 9% and 14% at 4 and 8 mg of TRF treatment, respectively. However, a significant reduction of 19% was observed at 12 mg of TRF treatment for 3 weeks when compared to saline-fed control. LDL-C showed a significant decline of 7%, 15% and 17% at 4, 8 and 12 mg of TRF treatment, respectively. No effect was seen on HDL-C levels at 3 doses of TRF, whereas HDL_2-C increased significantly by approximately 27% at 4, 8 and 12 mg of TRF administration. The decrease in HDL_3-C levels after 4, 8 and 12 mg of TRF treatment was 15%, 19% and 19%, respectively. ApoB levels showed a significant reduction of 23%, 25% and 25% at 4, 8 and 12 mg of TRF administration, respectively, whereas apoA-1 levels did not register any significant decrease at the 3 doses of TRF (Table 20). These results demonstrate that reduction in total cholesterol was due a significant decrease in LDL-C.

3.7.3 Regulation of hepatic HMG-CoA reductase activity and protein mass

Both HMG-CoA reductase activity and protein mass were significantly reduced, with a maximum decrease at 8 mg TRF (Table 21). Enzyme activity was decreased significantly by 48% at 4 mg TRF, 61% at 8 mg TRF and 62% decrease at 12 mg TRF. Similarly, protein mass of HMG-CoA reductase was reduced by 35%, 60% and 63% at 4, 8 and 12 mg TRF, respectively, when compared to saline-fed control (Table 21). These results demonstrate a differential effect of TRF at 3 doses of TRF on HMG-CoA reductase activity and protein mass with a optimal effect at 8 of TRF mg/kg/day.

TABLE 20

DOSE-DEPENDENT IMPACT OF TRF ON VLDL-C, LDL-C, HDL-C, HDL_2-C, HDL_3-C, ApoB AND ApoA-1 IN NORMOLIPIDEMIC RATS TREATED FOR 3 WEEKS

Parameters	NLP-C	NLP-T1	NLP-T2	NLP-T3
VLDL-cholesterol	8.6 ± 0.6*	7.8 ± 0.7 (-9.3%)	7.4 ± 0.5 (-13.9%)	7.0 ± 0.4 (-18.6%)¶
LDL-cholesterol	19.9 ± 0.7	18.5 ± 0.5 (-7.0%)¶	17.0 ± 0.7 (-14.6%)†	16.5 ± 0.5 (-17.1%)†
HDL-cholesterol	43.9 ± 0.9	43.1 ± 0.5 (-1.8%)	42.6 ± 0.8 (-3.0%)	42.2 ± 0.5 (-3.9%)
HDL_2-cholesterol	15.0 ± 0.7	18.9 ± 0.6 (+26.0%)‡	19.2 ± 0.6 (+28.0%)‡	19.0 ± 0.7 (+26.7%)‡
HDL_3-cholesterol	28.8 ± 0.3	24.5 ± 0.4 (-14.9%)‡	23.4 ± 0.2 (-18.7%)‡	23.2 ± 0.2 (-19.4%)†
ApoB	15.4 ± 0.6	11.9 ± 0.2 (-22.7%)‡	11.6 ± 0.6 (-24.7%)‡	11.6 ± 0.4 (-24.7%)‡
ApoA-1	90.7 ± 1.2	92.4 ± 1.7 (+1.9%)	93.2 ± 1.6 (+2.7%)	92.1 ± 2.3 (+1.5%)

*Values are mean (mg/dl) ± SD from plasma of 3 individual rats in each group.
NLP-C, saline-fed control; NLP-T1, NLP-T2 & NLP-T3, given 4 mg, 8 mg and 12 mg TRF/kg/ day, respectively, for 3 weeks.
Significantly different from NLP-C at $^{‡}p<0.001$, $^{†}p<0.01$ & $^{¶}p<0.05$.

TABLE 21

DOSE-DEPENDENT REGULATION OF ACTIVITY AND PROTEIN MASS OF HEPATIC HMG-CoA REDUCTASE IN NORMOLIPIDEMIC RATS TREATED WITH TRF FOR 3 WEEKS

Group	HMG-CoA reductase	
	Specific activity*	Protein mass**
NLP-C	7.88 ± 0.20	239 ± 10
NLP-T1	4.13 ± 0.16 (-47.6%)‡	156 ± 22 (-34.7%)‡
NLP-T2	3.04 ± 0.19 (-61.4%)‡	96 ± 4 (-59.8%)‡
NLP-T3	2.96 ± 0.18 (-62.4%)‡	89 ± 11 (-62.8%)‡

*Values are mean (nmoles of mevalonate formed/min/mg protein) ± SD from three separate assay in each group.

**Values are mean (μg/g of microsomal protein) ± SD from three separate assay in each group.

NLP-C, saline-fed control; NLP-T1, NLP-T2 & NLP-T3, given 4 mg, 8 mg and 12 mg TRF/kg/ day, respectively, for 3 weeks.

Significantly different from NLP-C at ‡$p<0.001$.

3.7.4 Impact on microsomal lipid peroxidation and LDL oxidation

As shown in Table 22 TRF administration to normolipidemic rats significantly prevented microsomal lipid peroxidation and LDL oxidation measured *in vitro* in a dose dependent manner. There was a significant decrease of 26% and 40% and 42% at 4, 8 and 12 mg TRF/kg/day in thiobarbituric acid reactive substances (TBARS) formation, in NLP-T1, NLP-T2 and NLP-T3, respectively, when compared to untreated control rats. Whereas, formation of conjugated dienes, a reflection of plasma oxidation was significantly decreased by approximately 15%, 32% and 36% in NLP-T1, NLP-T2 and NLP-T3 respectively, Table 22. These results demonstrate that TRF caused a dose-dependent antioxidant impact in normolipidemic rats with a maximum effect observed at 8 mg TRF/kg/day.

3.8 Dose-Dependent Impact of Purified Tocotrienol Rich Fraction on Plasma Lipids, Plasma Lipoproteins, ApoB, ApoA-1, Activity and Protein Mass of HMG-CoA Reductase and Microsomal Lipid Peroxidation and Plasma LDL Oxidation in Normolipidemic Rats After 3 Weeks of Treatment

In the experiments described below a dose-dependent effect of purified TRF on cholesterol dynamics in normolipidemic rats was investigated. Doses of **2.6, 5.2 and 7.8 mg purified TRF/kg/day** was administered to normolipidemic rats, which is equivalent to 4, 8 and 12 mg of TRF/kg/day, isolated from refined edible grade RBO.

3.8.1 Impact on plasma lipids

As depicted in Table 23, triglyceride concentration was decreased significantly by 23%, 30% and 34% at 2.6, 5.2 and 7.8 mg of purified TRF, respectively, after 3 weeks of treatment when compared to saline-fed normal control. No significant decrease in total cholesterol level at 2.6 mg of purified TRF was observed. However, a significant decline of 7% and 10% was seen at 5.2 and

TABLE 22

DOSE-DEPENDENT IMPACT OF TRF ON MICROSOMAL LIPID PEROXIDATION AND PLASMA LDL OXIDATION IN NORMOLIPIDEMIC RATS TREATED FOR 3 WEEKS

Group	TBARS*	Conjugated Diene**
NLP-C	2.23 ± 0.15	5.26 ± 0.45
NLP-T1	1.65 ± 0.13 (-26.0%)†	4.49 ± 0.37 (-14.6%)‡
NLP-T2	1.33 ± 0.12 (-40.3%)‡	3.60 ± 0.31 (-31.5%)‡
NLP-T3	1.30 ± 0.09 (-41.7%)‡	3.36 ± 0.30 (-36.1%)‡

*Values are mean (nmole/min/mg) ± SD of 4 separate assay in each group.
**Values are mean (nmole/min/ml) ± SD of 3 separate assay in each group.
NLP-C, saline-fed control; NLP-T1, NLP-T2 & NLP-T3, given 4 mg, 8 mg and 12 mg TRF/kg/ day, respectively, for 3 weeks.
Significantly different from NLP-C at $^{\ddagger}p<0.001$ and $^{\dagger}p<0.01$.

TABLE 23

DOSE-DEPENDENT EFFECT OF PURIFIED TRF ON TRIGLYCERIDES, TOTAL, ESTERIFIED AND FREE CHOLESTEROL IN NORMOLIPIDEMIC RATS AFTER 3 WEEKS OF TREATMENT

Parameters	NLP-C	NLP-T4	NLP-T5	NLP-T6
Triglycerides	51.2 ± 2.79*	39.5 ± 1.86 (-22.8%)‡	35.7 ± 1.2 (-30.3%)‡	34.0 ± 1.70 (-33.6%)‡
Total cholesterol	72.8 ± 1.76	69.4 ± .65 (-4.7%)	67.4 ± 1.4 (-7.4%)¶	65.7 ± 1.82 (-9.7%)†
Esterified cholesterol	53.0 ± 2.29	48.6 ± 1.45 (-8.3%)	49.3 ± 1.1 (-7.0%)	48.3 ± 2.3 (-8.9%)
Free cholesterol	19.9 ± 1.20	20.7 ± 0.71 (+4.0%)	18.1 ± 0.4 (-9.0%)	17.4 ± 0.6 (-12.6%)¶

**Values are mean (mg/dl) ± SD from plasma of 3 individual rats in each group.*
NLP-C, saline-fed control; NLP-T4, NLP-T5 & NLP-T6, given 2.6 mg, 5.2 mg and 7.8 mg purified TRF/kg/day, respectively, for 3 weeks.
Significantly different from NLP-C at ‡$p<0.001$, †$p<0.01$ & ¶$p<0.05$.

7.8 mg purified TRF, respectively. There was no significant effect on esterified cholesterol at all 3 doses of purified TRF. Free cholesterol did not show any significant change at 2.6 and 5.2 mg of purified TRF, but a significant decline of 13% at 7.8 mg of purified TRF treatment was observed (Table 23). These results demonstrate that three doses of purified TRF caused a differential decrease in lipid parameters after 3 weeks of treatment to normolipidemic rats.

3.8.2 Effect on plasma lipoprotein lipids, apoB and apoA-1

As shown in Table 24, administration of 2.6, 5.2 and 7.8 mg of purified TRF/kg/day was associated with no significant decline in VLDL-C, whereas LDL-C was decreased insignificantly by 6%, significantly by 16% and 18% at the above three doses of purified TRF, respectively. There was no change in HDL-C at all the 3 doses, whereas HDL_2-C showed a significant increase of 22%, 29% and 29% at 2.6, 5.2 and 7.8 mg purified TRF treatment, respectively. HDL_3-C subfraction was decreased significantly by 14%, 17% and 19%, at 2.6, 5.2 and 7.8 mg of purified TRF respectively, after 3 weeks of treatment. ApoB levels were significantly declined by 20%, 27% and 28% at 2.6, 5.2 and 7.8 mg of purified TRF treatment, receptively. No significant change in apoA-1 levels was observed (Table 24). These results demonstrate a significant decline in LDL-C and apoB levels at 2.6, 5.2 and 7.8 mg purified TRF/kg/day with a significant increase in HDL_2-C levels. In addition, a maximal hypolipidemic impact of TRF was obtained at 5.2 mg of purified TRF/kg/day which is equivalent to 8 mg of TRF/kg/day.

3.8.3 Regulation of hepatic HMG-CoA reductase activity and protein mass

Purified TRF showed a dose-dependent decline in HMG-CoA reductase activity and protein mass as shown in Table 25. Activity was decreased by 45%, 58% and 60% at 2.6, 5.2 and 7.8 mg purified TRF treatment, respectively, when compared to saline-fed control after 3 weeks. Similarly, protein mass was decreased by 41%, 57% and 60% at 2.6, 5.2 and 7.8 mg of purified TRF,

TABLE 24

DOSE-DEPENDENT IMPACT OF PURIFIED TRF ON VLDL-C, LDL-C, HDL-C, HDL_2-C, HDL_3-C, ApoB AND ApoA-1 IN NORMOLIPIDEMIC RATS AFTER 3 WEEKS OF TREATMENT

Parameters	NLP-C	NLP-T4	NLP-T5	NLP-T6
VLDL-cholesterol	8.6 ± 0.6*	7.9 ± 0.8 (-8.1%)	7.1 ± 0.5 (-17.4%)¶	7.6 ± 0.4 (-11.6%)
LDL-cholesterol	19.9 ± 0.7	18.8 ± 0.7 (-5.5%)	16.7 ± 0.6 (-16.1%)†	16.4 ± 0.6 (-17.6%)†
HDL-cholesterol	43.9 ± 0.9	43.1 ± 0.7 (-1.8%)	43.3 ± 0.8 (-1.4%)	42.7 ± 0.6 (-2.7%)
HDL_2-cholesterol	15.0 ± 0.7	18.3 ± 0.6 (+22.0%)‡	19.4 ± 0.5 (+29.3%)‡	19.3 ± 0.6 (+28.7%)‡
HDL_3-cholesterol	28.8 ± 0.3	24.7 ± 0.2 (-14.2%)‡	23.9 ± 0.3 (-17.0%)‡	23.4 ± 0.6 (-18.7%)‡
ApoB	15.4 ± 0.6	12.4 ± 0.6 (-19.5%)‡	11.2 ± 0.5 (-27.3%)‡	11.1 ± 0.7 (-27.9%)‡
ApoA-1	90.7 ± 1.2	92.3 ± 1.7 (+1.8%)	92.8 ± 1.8 (+2.3%)	91.7 ± 1.5 (+1.1%)

*Values are mean (mg/dl) ± SD from plasma of 3 individual rats in each group.
NLP-C, saline-fed control; NLP-T4, NLP-T5 & NLP-T6, given 2.6 mg, 5.2 mg and 7.8 mg purified TRF/kg/day, respectively, for 3 weeks.
Significantly different from NLP-C at $^{‡}p<0.001$, $^{†}p<0.01$ & $^{¶}p<0.05$.

TABLE 25

DOSE-DEPENDENT *IN VIVO* MODULATION OF ACTIVITY AND PROTEIN MASS OF HEPATIC HMG-CoA REDUCTASE IN NORMOLIPIDEMIC RATS TREATED WITH PURIFIED TRF FOR 3 WEEKS

Group	HMG-CoA reductase	
	Specific activity*	Protein mass**
NLP-C	7.88 ± 0.20	239 ± 10
NLP-T4	4.37 ± 0.52 (-44.5%)‡	140 ± 12 (-41.4%)‡
NLP-T5	3.30 ± 0.19 (-58.1%)‡	103 ± 9 (-56.9%)‡
NLP-T6	3.13 ± 0.24 (-60.3%)‡	96 ± 9 (-59.8%)‡

*Values are mean (nmoles of mevalonate formed/min/mg protein) ± SD from three separate assay in each group.

**Values are mean (μg/g of microsomal protein) ± SD from three separate assay in each group.

NLP-C, saline-fed control; NLP-T4, NLP-T5 & NLP-T6, given 2.6 mg, 5.2 mg and 7.8 mg purified TRF/kg/day, respectively, for 3 weeks.

Significantly different from NLP-C at ‡$p<0.001$.

respectively, after 3 weeks of treatment (Table 25). These results demonstrate that reduction in HMG-CoA reductase activity is due to decrease in its protein mass. The results also indicate that maximal reduction in HMG-CoA reductase activity and protein mass was mediated by 5.2 mg of purified TRF or 8 mg of TRF/kg/day.

3.8.4 Impact on microsomal lipid peroxidation and LDL oxidation

As shown in Table 26 TRF administration to normolipidemic rats significantly prevented microsomal lipid peroxidation and LDL oxidation measured *in vitro* in a dose dependent manner. There was a significant decrease of 28% and 42% and 43% at 2.6, 5.2 and 7.8 mg purified TRF/kg/day in thiobarbituric acid reactive substances (TBARS) formation, in NLP-T4, NLP-T5 and NLP-T6, respectively, when compared to untreated control rats. Whereas, formation of conjugated dienes, a reflection of plasma oxidation was significantly decreased by approximately 17%, 36% and 38% in NLP-T4, NLP-T5 and NLP-T6 respectively, Table 22. These results demonstrate that TRF caused a dose-dependent antioxidant impact in normolipidemic rats with a maximum effect observed at 5.2 mg purified or 8 mg of TRF/kg/day.

3.9 Dose-Dependent Impact of Purified Tocotrienol Rich Fraction on Plasma Lipids, Plasma Lipoproteins, ApoB, ApoA-1, Activity and Protein Mass of HMG-CoA Reductase and Microsomal Lipid Peroxidation and Plasma LDL Oxidation in Hyperlipidemic Rats Treated For 7 Days

As evident from the above combined results, the hypolipidemic impact of purified TRF in experimentally induced hyperlipidemic rats at the doses of 16.25 and 32.50 mg/kg/day, which is equivalent to 25 and 50 mg TRF/kg/day, after 5 and 7 days of treatment was quite similar. Therefore, to find out the optimal dose of purified TRF in terms of its cholesterol lowering efficiency, **2.6, 5.2 and 7.8 mg of purified TRF/kg/day**, which is equivalent to 4, 8 and 12 mg TRF/kg/day, was administered to experimentally induced hyperlipidemic rats.

TABLE 26

DOSE-DEPENDENT IMPACT OF PURIFIED TRF ON MICROSOMAL LIPID PEROXIDATION AND PLASMA LDL OXIDATION IN NORMOLIPIDEMIC RATS TREATED FOR 3 WEEKS

Group	TBARS*	Conjugated Diene**
NLP-C	2.23 ± 0.15	5.26 ± 0.45
NLP-T4	1.60 ± 0.11 (-28.2%)†	4.36 ± 0.30 (-17.1%)‡
NLP-T5	1.29 ± 0.11 (-42.1%)‡	3.38 ± 0.31 (-35.7%)‡
NLP-T6	1.27 ± 0.08 (-43.0%)‡	3.24 ± 0.28 (-38.4%)‡

*Values are mean (nmole/min/mg) ± SD of 4 separate assay in each group.
**Values are mean (nmole/min/ml) ± SD of 3 separate assay in each group.
NLP-C, saline-fed control; NLP-T4, NLP-T5 & NLP-T6, given 2.6 mg, 5.2 mg and 7.8 mg purified TRF/kg/day, respectively, for 3 weeks.
Significantly different from NLP-C at $^{\ddagger}p<0.001$ and $^{\dagger}p<0.01$.

3.9.1 Effect on plasma lipids

Atherogenic diet had a profound impact on the lipid parameters of the hyperlipidemic rats in comparison to the normal rats. As seen in Table 27, triglycerides, total cholesterol, esterified cholesterol and free cholesterol were increased from 51, 73, 53 and 20 mg/dl, respectively in NLP-C to 156, 175, 141 and 34 mg/dl in HLP-C, respectively. Seven days after the withdrawal of atherogenic diet a significant decrease in triglycerides, total cholesterol, esterified cholesterol and free cholesterol levels to 139, 139, 109 and 29 mg/dl, respectively, in HLP-UC was observed. However, administration of 2.6, 5.2 and 7.8 mg of purified TRF /kg/day for 7 days significantly reduced these parameters at a faster rates. When compared to saline-fed hyperlipidemic control (HLP-UC), triglycerides decreased by 26%, 30% and 32% in HLP-T1, HLP-T2 and HLP-T3, respectively. Compared to HLP-UC, total cholesterol was reduced by 10% at 2.6 mg purified TRF, whereas it was reduced by 24% and 30% at 5.2 and 7.8 mg purified TRF, respectively. Similarly, esterified cholesterol showed a decline of 11% in HLP-T1, 27% in HLP-T2 and 31% in HLP-T3. Free cholesterol was insignificantly reduced by 5% in HLP-T1, whereas it was significantly declined by 14% and 27% in HLP-T2 and HLP-T3, respectively (Table 27). These results demonstrate that maximal anticholesterol effect on plasma lipids of hyperlipidemic rats was exerted at 5.2 mg of purified TRF, which is equivalent 8 mg TRF/kg/day.

3.9.2 Impact on plasma lipoproteins lipids, apoB and apoA-1

As shown in Table 28, all the plasma lipoproteins along with apoB and apoA-1 were increased on feeding atherogenic diet for three weeks. However, a reduction in these parameters was observed 7 days after the withdrawal of atherogenic diet. VLDL-C, LDL-C and apoB levels were affected most after the administration of TRF for 7 days. When compared to saline-fed hyperlipidemic control (HLP-UC), HLP-T1, HLP-T2 and HLP-T3 showed a significant decline in

TABLE 27

DOSE-DEPENDENT EFFECT OF PURIFIED TRF ON TRIGLYCERIDES, TOTAL, ESTERIFIED AND FREE CHOLESTEROL IN HYPERLIPIDEMIC RATS AFTER 7 DAYS OF ADMINISTRATION

Parameters	NLP-C	HLP-C	HLP-UC	HLP-T1	HLP-T2	HLP-T3
Triglycerides	51.2 ± 2.79*	156.5 ± 2.65	139.4 ± 2.60	103.7 ± 2.76 (-25.6%)‡	97.1 ± 2.07 (-30.3%)‡	95.0 ± 2.60 (-31.8%)‡
Total cholesterol	72.8 ± 1.76	174.7 ± 3.15	138.7 ± 1.70	124.5 ± 1.86 (-10.2%)‡	104.7 ± 2.00 (-24.5%)‡	96.6 ± 1.71 (-30.3%)‡
Esterified cholesterol	53.0 ± 2.29	141.4 ± 2.2	109.0 ± 1.95	96.6 ± 1.03 (-11.4%)‡	79.1 ± 1.75 (-27.4%)‡	75.0 ± 1.25 (-31.2%)‡
Free cholesterol	19.9 ± 1.20	33.7 ± 0.55	29.4 ± 0.45	27.8 ± 1.27 (-5.4%)	25.3 ± 1.36 (-13.9%)‡	21.6 ± 0.47 (-26.5%)‡

*Values are mean (mg/dl) ± SD from plasma of 3 individual rats in each group.
HLP-C, hyperlipidemic control; HLP-UC, saline-fed control; HLP-T1, HLP-T2 & HLP-T3, given 2.6 mg, 5.2 mg and 7.8 mg purified TRF/kg/day, respectively, for 7 days.
Significantly different from HLP-UC at ‡$p<0.001$.

TABLE 28

DOSE-DEPENDENT IMPACT OF PURIFIED TRF ON VLDL-C, LDL-C, HDL-C, HDL_2-C, HDL_3-C, ApoB AND ApoA-1 IN HYPERLIPIDEMIC RATS TREATED FOR 7 DAYS

Parameters	NLP-C	HLP-C	HLP-UC	HLP-T1	HLP-T2	HLP-T3
VLDL-cholesterol	8.6 ± 0.6	16.3 ± 0.65*	13.1 ± 0.62	10.7 ± 0.61 (-18.3%)†	9.3 ± 0.90 (-29.0%)†	9.0 ± 0.65 (-31.3%)†
LDL-cholesterol	19.9 ± 0.7	108.0 ± 1.27	82.2 ± 1.94	69.2 ± 1.32 (-15.8%)‡	52.1 ± 1.42 (-36.6%)‡	42.8 ± 1.12 (-47.9%)‡
HDL-cholesterol	43.9 ± 0.9	50.8 ± 2.55	45.6 ± 1.30	44.8 ± 1.92 (-1.7%)	43.7 ± 1.20 (-4.2%)	45.5 ± 1.61 (-0.2%)
HDL_2-cholesterol	15.0 ± 0.7	20.4 ± 1.20	19.2 ± 0.76	17.6 ± 1.53 (-8.3%)	18.6 ± 1.11 (-3.1%)	18.3 ± 1.10 (-4.7%)
HDL_3-cholesterol	28.8 ± 0.3	30.4 ± 1.36	26.4 ± 0.56	27.5 ± 1.04 (+4.2%)	25.1 ± 0.62 (-4.9%)	27.5 ± 2.20 (+4.2%)
ApoB	15.4 ± 0.6	53.9 ± 1.72	45.5 ± 1.32	34.2 ± 1.97 (-24.8%)‡	20.4 ± 2.25 (-55.2%)‡	20.9 ± 1.30 (-54.1%)‡
ApoA-I	90.7 ± 1.2	101.8 ± 1.56	97.3 ± 1.16	96.3 ± 0.95 (-1.0%)	94.5 ± 1.35 (-2.9%)	93.1 ± 1.90 (-4.3%)¶

*Values are mean (mg/dl) ± SD from plasma of 3 individual rats in each group.
HLP-C, hyperlipidemic control; HLP-UC, saline-fed control; HLP-T1, HLP-T2 & HLP-T3, given 2.6 mg, 5.2 mg and 7.8 mg purified TRF/kg/day, respectively, for 7 days.
Significantly different from HLP-C from HLP-C at $^{\ddagger}p<0.001$, $^{\dagger}p<0.01$ & $^{\P}p<0.05$.

VLDL-C by 18%, 29.0% and 31%, whereas LDL-C was reduced by 16%, 37% and 48%, respectively. HDL-C, HDL_2-C, HDL_3-C levels were not affected significantly in HLP-T1, HLP-T2 and HLP-T3. ApoB also showed a significant decrease of 25%, 55% and 54%, whereas apoA-1 showed no significant effect at 2.6 mg and 5.2 mg but a significant decline of 4.3% ($p<0.05$) at 7.8 mg of purified TRF in HLP-T1, HLP-T2 and HLP-T3, respectively, when compared to HLP-UC (Table 28). These results demonstrate that the increase in the total cholesterol by feeding an atherogenic diet for three weeks is due to the significant increase of VLDL-C and LDL-C. As expected, feeding of atherogenic diet also results in an increase of apoB associated with LDL. However, administration of purified TRF was associated with a significant decrease in these lipoprotein lipids including apoB, with an optimal effect at 5.2 mg purified TRF, which is equivalent to 8 mg TRF/kg/day.

3.9.3 Regulation of hepatic HMG-CoA reductase activity and protein mass

As seen in Table 29, there was a significant reduction in both HMG-CoA reductase activity and protein mass in HLP-C as compared to NLP-C. The specific activity of HMG-CoA reductase in NLP-C was 7.88 units/mg, whereas it was decreased to 1.97 units/mg in HLP-C. Similarly, protein mass was decreased from 239 μg/g of microsomal protein in NLP-C to 102 μg/g of microsomal protein in HLP-C. Seven days after the withdrawal of the atherogenic diet, however, resulted in the increase in both the activity as well as the protein mass of HMG-CoA reductase. Administration of purified TRF to hyperlipidemic rats for 7 days resulted in further decrease of HMG-CoA reductase activity and protein mass by 36% and 15% in HLP-T1, 54% and 45% in HLP-T2 and 57% and 49% in HLP-T3, respectively (Table 29). These results demonstrate that feeding of atherogenic diet to normolipidemic rats significantly decrease the HMG-CoA reductase activity by negative feed-back regulation mediated via decrease in

TABLE 29

DOSE-DEPENDENT REGULATION OF ACTIVITY AND PROTEIN MASS OF HEPATIC HMG-CoA REDUCTASE IN HYPERLIPIDEMIC RATS AFTER 7 DAYS OF PURIFIED TRF ADMINISTRATION

Group	HMG-CoA Reductase	
	Specific activity*	Protein mass**
NLP-C	7.88 ± 0.20	239 ± 10
HLP-C	1.97 ± 0.19	102 ± 11
HLP-UC	5.74 ± 0.22	165 ± 15
HLP-T1	3.69 ± 0.27 (-35.7%)‡	140 ± 6 (-15.1%)
HLP-T2	2.65 ± 0.15 (-53.8%)‡	91 ± 9 (-44.8%)‡
HLP-T3	2.48 ± 0.28 (-56.8%)‡	85 ± 8 (-48.5%)‡

*Values are mean (nmoles of mevalonate formed/min/mg protein) ± SD from three separate assay in each group.

**Values are mean (μg/g of microsomal protein) ± SD from three separate assay in each group.

NLP-C, normal control; HLP-C, hyperlipidemic control; HLP-UC, saline-fed control; HLP-T1, HLP-T2 & HLP-T3, given 2.6 mg, 5.2 mg and 7.8 mg purified TRF/kg/day, respectively, for 7 days.

Significantly different from HLP-UC at ‡$p<0.001$.

HMG-CoA reductase protein mass. Feeding of TRF causes a further decline in HMG-CoA reductase activity by enhancing the decrease in its protein mass.

3.9.4 Effect on microsomal lipid peroxidation and plasma LDL oxidation

As shown in Table 30, formation of TBARS in microsomes was increased from 2.23 units/mg in NLP-C to 3.98 units/mg in HLP-C, whereas LDL oxidation in plasma increased from 5.26 units/ml in NLP-C to 9.35 units/ml in HLP-C, after feeding atherogenic diet for three weeks. These values were reduced to 2.49 units/mg and 7.45 units/ml in HLP-UC after 7 days of the withdrawal of atherogenic diet, but the decrease was enhanced significantly in purified TRF treated hyperlipidemic rats for 7 days. Lipid peroxidation in microsomes decreased by 26%, 39% and 40% in HLP-T1, HLP-T2 and HLP-T3, respectively, whereas formation of conjugated dienes in plasma was reduced by 38%, 50% and 53%, respectively (Table 30). These results demonstrate that the induction of hyperlipidemia in rats is associated with a significant increase in the microsomal lipid peroxidation as well as plasma LDL oxidation, which is significantly prevented by the administration of purified TRF.

Based on the above combined results and similar investigations related to hypolipidemic action of TRF in hypercholesterolemic patients carried out in our laboratory, an optimal dose of 8 mg TRF/kg/day and a 4.3% TRF yield of refined edible grade RBO, intake of an equivalent amount of 186 mg dietary refined RBO/kg/day should exert similar hypolipidemic impacts.

TABLE 30

DOSE-DEPENDENT IMPACT OF PURIFIED TRF ON MICROSOMAL LIPID PEROXIDATION AND PLASMA LDL OXIDATION IN HYPERLIPIDEMIC RATS TREATED FOR 7 DAYS

Group	TBARS*	Conjugated Diene**
NLP-C	2.23 ± 0.15	5.26 ± 0.45
HLP-C	3.98 ± 0.17	9.35 ± 0.63
HLP-UC	2.49 ± 0.16	7.45 ± 0.54
HLP-T1	1.83 ± 0.13 (-26.5%)†	4.61 ± 0.25 (-38.1%)‡
HLP-T2	1.54 ± 0.13 (-38.5%)‡	3.72 ± 0.28 (-50.1%)‡
HLP-T3	1.49 ± 0.14 (-40.2%)‡	3.54 ± 0.29 (-52.5%)‡

*Values are mean (nmole/min/mg) ± SD of 4 separate assay in each group.
**Values are mean (nmole/min/ml) ± SD of 3 separate assay in each group.
NLP-C, normal control; HLP-C, hyperlipidemic control; HLP-UC, saline-fed control; HLP-T1, HLP-T2 & HLP-T3, given 2.6 mg, 5.2 mg and 7.8 mg purified TRF/kg/day, respectively, for 7 days.
Significantly different from HLP-UC at $^{\ddagger}p<0.001$ and $^{\dagger}p<0.01$.

3.10 Dose-Dependent Effect of Purified Tocotrienol Rich Fraction Isolated from the Raw Bran of Basmati on Plasma Lipids, Plasma Lipoproteins, ApoB, ApoA-1, Activity and Protein Mass of HMG-CoA Reductase and Microsomal Lipid Peroxidation and Plasma LDL Oxidation in Hyperlipidemic Rats After 7 Days of Treatment

Earlier reports indicated that rice bran or rice bran oil (RBO) vary in their hypocholesterolemic activity and some have shown that neither rice bran nor RBO lowered cholesterol levels. These findings may be explained by other reports that some, but not all, rice cultivars contain tocotrienols (T_3), which exert a powerful hypocholesterolemic action. Because of these variation in types and content of T_3, one finds variation in the cholesterol lowering effects of RBO isolated from different cultivars of rice. Out of α-, β-, γ- and δ-T_3, δ-form was most potent in terms of cholesterol lowering effect, followed by γ-form. α- Tocotrienol has very low effect, whereas, β-form has no anti-cholesterol activity. Based on the above optimal dose of 8 mg TRF/kg/day, we have investigated the hypolipidemic impacts of purified TRF isolated from two cultivars of rice, raw basmati and saket-4, in a linear range of 3 and 6 mg TRF/kg/day. As seen in Table 31, the combined percent content of γ- and δ-T_3 in each cultivar was adjusted equivalent to that present in 3 and 6 mg of TRF isolated from refined edible grade RBO. Hence, based on 12.4% of combined concentration of γ- and δ-T_3 quantified in TRF of refined RBO, equivalent calculated doses of purified TRF from raw Basmati, containing 22.2% of γ- and δ-T_3, was 1.67 and 3.35 mg. Similarly, equivalent calculated doses of purified TRF from raw Saket-4, containing 31.8% of γ- and δ-T_3, was 1.17 and 2.34 mg (Table 31). Therefore, impact of **1.67 and 3.35 mg/kg/day** of purified TRF from raw Basmati and **1.17 and 2.34 mg/kg/day** of purified TRF from raw Saket-4 was investigated in hyperlipidemic rats after 7 days of treatment.

TABLE 31

CALCULATED EQUIVALENT DOSE OF PURIFIED TRF, FROM TWO CULTIVARS OF RICE, BASED ON OPTIMAL ANTICHOLESTEROL ACTIVITY OF γ- AND δ-TOCOTRIENOLS PRESENT IN TRF ISOLATED FROM EDIBLE GRADE REFINED RBO

Type	γ- and δ-T_3	3mg TRF*	6 mg TRF	8 mg TRF
Refined RBO	12.4	3.00	6.00	8.00
Raw Basmati	22.2	1.67	3.35	4.47
Raw Saket-4	31.8	1.17	2.34	3.12
Parboiled Mansuri	16.2	2.30	4.60	6.12

* Values of equivalent doses are calculated on the basis of total content of 12.4% γ- and δ-T_3, present in TRF isolated from edible grade refined RBO.

3.10.1 Impact on plasma lipids

Table 32 shows the impact of purified TRF on plasma lipids of hyperlipidemic rats treated for 7 days. All the lipid parameters were significantly increased after the feeding of atherogenic diet for three weeks. Triglycerides increased from 54 to 162 mg/dl, total cholesterol increased from 73 to 183 mg/dl, esterified cholesterol increased from 53 to 144 mg/dl and free cholesterol increased from 20 to 39 mg/dl. There was a decline in triglycerides, total cholesterol, esterified cholesterol and free cholesterol by 16%, 21%, 21% and 20%, respectively, in HLP-UC after 7 days of withdrawal of atherogenic diet, as compared to values in HLP-C. However, due to purified TRF treatment, a further significant reduction in these lipid parameters was observed. Triglycerides were reduced by 21% and 27% in HLP-T1 and HLP-T2, respectively. Total cholesterol showed a decrease of 13% and 27% at 1.67 and 3.35 mg purified TRF, respectively, when compared to saline-fed hyperlipidemic control rats. Similarly, esterified cholesterol registered a decrease of 12% and 28% in HLP-T1 and HLP-T2, respectively. There was also a significant reduction of 14% and 20% in free cholesterol of HLP-T1 and HLP-T2, respectively (Table 32). These results indicate that purified TRF mediate a differential dose-dependent decrease in the levels of cholesterol.

3.10.2 Effect on plasma lipoproteins, lipids, apoB and apoA-1

As seen in Table 33, plasma lipoproteins along with apoB and apoA-1 were also significantly elevated after the feeding of atherogenic diet. These parameters were decreased 7 days after the withdrawal of atherogenic diet. However, the decrease in VLDL-C (14%) and LDL-C (31%) was highly significant in purified TRF treated HLP-T1 and HLP-T2 groups, when compared to HLP-UC. LDL-C levels were reduced by 19% and 38% in HLP-T1 and HLP-T2, respectively. HDL-C, HDL_2-C and HDL_3-C levels did not show any significant change

TABLE 32

DOSE-DEPENDENT EFFECT OF PURIFIED TRF ISOLATED FROM BRAN OF BASMATI ON TRIGLYCERIDES, TOTAL, ESTERIFIED AND FREE CHOLESTEROL IN HYPERLIPIDEMIC RATS AFTER 7 DAYS OF ADMINISTRATION

Parameters	NLP-C	HLP-C	HLP-UC	HLP-T1	HLP-T2
Triglycerides	53.6 ± 2.44*	162.2 ± 5.19	135.5 ± 4.08	107.6 ± 3.46 (-20.6%)‡	98.4 ± 2.38 (-27.4%)‡
Total cholesterol	73.0 ± 1.86	183.4 ± 6.36	145.2 ± 4.18	127.1 ± 4.06 (-12.5%)‡	106.7 ± 3.24 (-26.5%)‡
Esterified cholesterol	52.5 ± 2.12	144.3 ± 4.12	114.4 ± 3.06	100.6 ± 3.08 (-12.1%)‡	82.0 ± 2.87 (-28.3%)‡
Free cholesterol	20.5 ± 1.38	39.1 ± 2.33	30.8 ± 1.38	26.5 ± 1.10 (-14.0%)†	24.7 ± 1.68 (-19.8)†

*Values are mean (mg/dl) ± SD from plasma of 4 individual rats in each group.
NLP-C, normal control; HLP-C, hyperlipidemic control; HLP-UC, saline-fed control; HLP-T1 & HLP-T2, given 1.67 mg and 3.35 mg of purified TRF/kg/day, respectively, for 7 days.
Significantly different from HLP-UC at ‡$p<0.001$ & †$p<0.01$.

TABLE 33

DOSE-DEPENDENT IMPACT OF PURIFIED TRF ISOLATED FROM BRAN OF BASMATI ON VLDL-C, LDL-C, HDL-C, HDL_2-C, HDL_3-C, ApoB AND ApoA-1 IN HYPERLIPIDEMIC RATS AFTER 7 DAYS OF ADMINISTRATION

Parameters	NLP-C	HLP-C	HLP-UC	HLP-T1	HLP-T2
VLDL-cholesterol	8.6 ± 0.62*	16.9 ± 1.72	13.8 ± 1.11	11.9 ± 1.29 (-13.8%)	9.5 ± 0.58 (-31.2%)‡
LDL-cholesterol	21.3 ± 1.74	114.1 ± 4.57	88.0 ± 2.70	71.0 ± 2.88 (-19.3%)‡	54.8 ± 1.93 (-37.7%)‡
HDL-cholesterol	44.3 ± 2.31	51.8 ± 4.44	44.4 ± 2.17	44.9 ± 2.49 (+1.1%)	43.7 ± 2.44 (-1.6%)
HDL_2-cholesterol	15.6 ± 1.60	21.1 ± 2.22	17.6 ± 1.40	18.4 ± 0.92 (+4.5%)	18.5 ± 1.01 (+5.1%)
HDL_3-cholesterol	28.6 ± 1.70	30.6 ± 2.30	26.8 ± 1.70	26.5 ± 1.84 (-1.1%)	25.2 ± 1.44 (-6.0%)
ApoB	16.7 ± 1.20	59.4 ± 2.72	43.7 ± 2.76	37.2 ± 1.62 (-14.9%)†	23.0 ± 1.91 (-47.4%)‡
ApoA-I	92.8 ± 2.31	106.1 ± 3.66	98.8 ± 3.04	97.4 ± 2.40 (-1.4%)	94.8 ± 3.03 (-4.0%)

*Values are mean (mg/dl) ± SD from plasma of 4 individual rats in each group.
NLP-C, normal control; HLP-C, hyperlipidemic control; HLP-UC, saline-fed control; HLP-T1 & HLP-T2, given 1.67 mg and 3.35 mg of purified TRF/kg/day, respectively, for 7 days.
Significantly different from HLP-UC at ‡$p<0.001$ & †$p<0.01$.

compared to saline-fed control rats. ApoB was significantly reduced by 15% in HLP-T1, but decreased highly significantly in HLP-T2 by 47%. Again, apoA-1 did not show any significant change (Table 33). These results demonstrate that feeding of atherogenic diet causes a significant increase of VLDL-C and LDL-C which in turn increase the level of total cholesterol. As evident, feeding of atherogenic diet also results in a concomitant increase of apoB which is associated with LDL. However, administration of purified TRF substantially decrease these lipid parameters including apoB.

3.10.3 Modulation of hepatic HMG-CoA reductase activity and protein mass

Feeding of atherogenic diet for three weeks to rats caused a significant decline in both enzyme activity as well as protein mass of HMG-CoA reductase. As shown in Table 34, specific activity was decreased from 8.20 units/mg in NLP-C to 1.90 units/mg in HLP-C, whereas protein mass decreased from 239 μg/g of microsomal protein in NLP-C to 102 μg/g of microsomal protein in HLP-C. These parameters were increased after the withdrawal of atherogenic diet. However, administration of purified TRF for 7 days prevented the increase in enzyme activity by 37% in HLP-T1 and 54% in HLP-T2, whereas enzyme mass was decreased by 28% and 38% in HLP-T1 and HLP-T2, respectively, when compared to HLP-UC (Table 34). These results indicate that feeding of exogenous cholesterol or purified TRF to rats decrease the synthesis of endogenous cholesterol via decrease in HMG-CoA reductase activity which in turn is mediated by the decrease in its protein mass. However, withdrawal of atherogenic diet relieves this inhibition which is evident from the increase in HMG-CoA reductase activity as well as its protein mass.

TABLE 34

DOSE-DEPENDENT *IN VIVO* MODULATION OF ACTIVITY AND PROTEIN MASS OF HEPATIC HMG-CoA REDUCTASE IN HYPERLIPIDEMIC RATS TREATED WITH PURIFIED TRF ISOLATED FROM BRAN OF BASMATI AFTER 7 DAYS OF ADMINISTRATION

Group	HMG-CoA Reductase	
	Specific activity*	Protein mass**
NLP-C	8.20 ± 0.32	239 ± 10
HLP-C	1.90 ± 0.15	102 ± 11
HLP-UC	5.91 ± 0.25	165 ± 15
HLP-T1	3.75 ± 0.21 (-36.5%)‡	119 ± 6 (-27.9%)‡
HLP-T2	2.74 ± 0.18 (-53.6%)‡	103 ± 4 (-37.6%)‡

*Values are mean (nmoles of mevalonate formed/min/mg protein) ± SD from three separate assay in each group.

**Values are mean (μg/g of microsomal protein) ± SD from three separate assay in each group.

NLP-C, normal control; HLP-C, hyperlipidemic control; HLP-UC, saline-fed control; HLP-T1 & HLP-T2, given 1.67 mg and 3.35 mg of purified TRF/kg/day, respectively, for 7 days.

Significantly different from HLP-UC at ‡$p<0.001$, †$p<0.01$ & ¶$p<0.05$.

3.10.4 Effect on microsomal lipid peroxidation and plasma LDL oxidation

As shown in Table 35, formation of TBARS in microsomes was increased from 2.41 units/mg in NLP-C to 4.14 units/mg in HLP-C, whereas LDL oxidation in plasma increased from 5.31 units/ml in NLP-C to 10.17 units/ml in HLP-C, after feeding atherogenic diet for three weeks. These values were reduced to 2.81 units/mg and 7.78 units/ml in HLP-UC after 7 days of the withdrawal of atherogenic diet, but the decrease was enhanced significantly in purified TRF treated hyperlipidemic rats for 7 days. Lipid peroxidation in microsomes decreased by 35% and 47% in HLP-T1 and HLP-T2, respectively, whereas formation of conjugated dienes in plasma was reduced by 36% and 46%, respectively (Table 35). These results demonstrate that the induction of hyperlipidemia in rats is associated with a significant increase in the microsomal lipid peroxidation as well as plasma LDL oxidation, which is significantly prevented by the administration of purified TRF.

3.11 Dose-Dependent Impact of Purified Tocotrienol Rich Fraction Isolated from the Raw Bran of Saket-4 on Plasma Lipids, Lipoproteins Lipids, ApoB and ApoA-1, Activity and Protein Mass of HMG-CoA Reductase, Microsomal Lipid Peroxidation and Plasma LDL Oxidation in Hyperlipidemic Rats After 7 Days of Administration

3.11.1 Effect on plasma lipids

Similar to results summarized in Table 36, there was an increase in the lipid parameters of hyperlipidemic rats after the feeding of atherogenic diet for three weeks, which showed a substantial decline after 7 days of withdrawal of atherogenic diet. However, these parameters further declined in rats treated with purified TRF, which was isolated from the bran of Saket-4. Compared to saline-fed hyperlipidemic control (HLP-UC) rats, the decrease in triglycerides was 24% and 28% in HLP-T3 and HLP-T4, respectively. Total cholesterol showed a dose-dependent decrease of 11% at 1.17 mg purified TRF and 24% at 2.34 mg purified

TABLE 35

DOSE-DEPENDENT IMPACT OF PURIFIED TRF ISOLATED FROM BASMATI ON MICROSOMAL LIPID PEROXIDATION AND PLASMA LDL OXIDATION IN HYPERLIPIDEMIC RATS TREATED FOR 7 DAYS

Group	TBARS*	Conjugated Diene**
NLP-C	2.41 ± 0.15	5.31 ± 0.40
HLP-C	4.14 ± 0.23	10.17 ± 0.58
HLP-UC	2.81 ± 0.18	7.78 ± 0.43
HLP-T1	1.82 ± 0.13 (-35.2%)†	5.02 ± 0.27 (-35.5%)‡
HLP-T2	1.50 ± 0.11 (-46.6%)‡	4.18 ± 0.33 (-46.3%)‡

*Values are mean (nmole/min/mg) ± SD of 4 separate assay in each group.
**Values are mean (nmole/min/ml) ± SD of 3 separate assay in each group.
NLP-C, normal control; HLP-C, hyperlipidemic control; HLP-UC, saline-fed control; HLP-T1 & HLP-T2, given 1.67 mg and 3.35 mg of purified TRF/kg/day, respectively, for 7 days.
Significantly different from HLP-UC at $^{\ddagger}p<0.001$ and $^{\dagger}p<0.01$.

TABLE 36

DOSE-DEPENDENT EFFECT OF PURIFIED TRF ISOLATED FROM BRAN OF SAKET-4 ON TRIGLYCERIDES, TOTAL, ESTERIFIED AND FREE CHOLESTEROL IN HYPERLIPIDEMIC RATS TREATED FOR 7 DAYS

Parameters	NLP-C	HLP-C	HLP-UC	HLP-T3	HLP-T4
Triglycerides	53.6 ± 2.44*	162.2 ± 5.19	135.5 ± 4.08	102.8 ± 3.55 (-24.1%)‡	97.1 ± 3.31 (-28.3%)‡
Total cholesterol	73.0 ± 1.86	183.4 ± 6.36	145.2 ± 4.18	130.6 ± 4.70 (-10.5%)†	110.8 ± 4.24 (-23.7%)‡
Esterified cholesterol	52.5 ± 2.12	144.3 ± 4.12	114.4 ± 3.06	103.9 ± 3.39 (-9.2%)†	84.7 ± 3.25 (-26.0%)‡
Free cholesterol	20.5 ± 1.38	39.1 ± 2.33	30.8 ± 1.38	26.7 ± 1.46 (-13.3%)†	26.1 ± 1.06 (-15.2%)†

*Values are mean (mg/dl) ± SD from plasma of 4 individual rats in each group.
NLP-C, normal control; HLP-C, hyperlipidemic control; HLP-UC, saline-fed control; HLP-T3 & HLP-T4, given 1.17 mg and 2.34 mg of purified TRF/kg/day, respectively, for 7 days.
Significantly different from HLP-UC at ‡$p<0.001$ & †$p<0.01$.

TRF/kg/day. Similarly, esterified cholesterol decreased by 9% and 26% in HLP-T3 and HLP-T4, respectively, when compared to HLP-UC. Free cholesterol also showed a marked decline of 13% and 15% in HLP-T3 and HLP-T4, respectively (Table 36). These results demonstrate that purified TRF mediate a dose-dependent linear decrease in total and esterified cholesterol with a non-linear decrease in the levels of triglycerides and free cholesterol.

3.11.2 Impact on plasma lipoproteins lipids, apoB and apoA-1

As shown in Table 37, a significant increase in the plasma lipoprotein lipids as well as apoB and apoA-1 was observed after the induction of hyperlipidemia in rats fed atherogenic diet for three weeks. Seven days after the withdrawal of atherogenic diet a significant decline in these parameters were observed. However, the reduction was significantly greater in VLDL-C, LDL-C and apoB in purified TRF treated rats. In comparison to HLP-UC, VLDL-C decreased by 36% in HLP-T3 and 25% in HLP-T4. LDL-C showed a dose-dependent decrease of 15% at 1.17 mg purified TRF and 36% at 2.34 mg purified TRF /kg/day. HDL-C, HDL_2-C and HDL_3-C did not show any significant change after the administration of purified TRF. ApoB was reduced by 12% in HLP-T3, whereas it declined by 39% in HLP-T4. On the other hand, apoA-1 showed no decline in HLP-T3 and a significant decrease of 6% ($p<0.05$) in HLP-T4, when compared to saline-fed hyperlipidemic control rats (Table 37). These results indicate that purified TRF has a significant effect on the levels of VLDL-C, LDL-C and apoB in hyperlipidemic rats. On the other hand, it does not affect the levels of HDL-C or its sub-fractions, HDL_2-C and HDL_3-C.

3.11.3 Modulation of hepatic HMG-CoA reductase activity and protein mass

As seen in Table 38, consistent with the data presented in Table 30, a severe decline in the activity and protein mass of HMG-CoA reductase in HLP-C rats was observed, which began to increase 7 days after the removal of atherogenic

TABLE 37

DOSE-DEPENDENT IMPACT OF PURIFIED TRF ISOLATED FROM BRAN OF SAKET-4 ON VLDL-C, LDL-C, HDL-C, HDL_2-C, HDL_3-C, ApoB AND ApoA-1 IN HYPERLIPIDEMIC RATS AFTER 7 DAYS OF ADMINISTRATION

Parameters	NLP-C	HLP-C	HLP-UC	HLP-T3	HLP-T4
VLDL-cholesterol	8.6 ± 0.62*	16.9 ± 1.72	13.8 ± 1.11	8.8 ± 0.80 (-36.2%)‡	10.3 ± 1.11 (-25.4%)†
LDL-cholesterol	21.3 ± 1.74	114.1 ± 4.57	88.0 ± 2.70	74.7 ± 3.13 (-15.1%)‡	56.7 ± 1.54 (-35.6%)‡
HDL-cholesterol	44.3 ± 2.31	51.8 ± 4.44	44.4 ± 2.17	46.1 ± 2.20 (+3.8%)	44.2 ± 2.03 (-0.4%)
HDL_2-cholesterol	15.6 ± 1.60	21.1 ± 2.22	17.6 ± 1.40	18.4 ± 0.87 (+4.5%)	17.7 ± 0.97 (+0.6%)
HDL_3-cholesterol	28.6 ± 1.70	30.6 ± 2.30	26.8 ± 1.70	27.7 ± 1.37 (+3.3%)	26.4 ± 1.50 (-1.5%)
ApoB	16.7 ± 1.20	59.4 ± 2.72	43.7 ± 2.76	38.5 ± 2.21 (-11.9%)¶	26.8 ± 1.22 (-38.7%)‡
ApoA-I	92.8 ± 2.31	106.1 ± 3.66	98.8 ± 3.04	98.8 ± 3.27 (0.0%)	92.8 ± 2.70 (-6.1%)¶

*Values are mean (mg/dl) ± SD from plasma of 4 individual rats in each group.
NLP-C, normal control; HLP-C, hyperlipidemic control; HLP-UC, saline-fed control; HLP-T3 & HLP-T4, given 1.17 mg and 2.34 mg of purified TRF/kg/day, respectively, for 7 days.
Significantly different from HLP-UC at $^{\ddagger}p<0.001$, $^{\dagger}p<0.01$ & $^{\P}p<0.05$.

TABLE 38

DOSE-DEPENDENT REGULATION OF ACTIVITY AND PROTEIN MASS OF HEPATIC HMG-CoA REDUCTASE IN HYPERLIPIDEMIC RATS TREATED WITH PURIFIED TRF ISOLATED FROM BRAN OF SAKET-4 FOR 7 DAYS

Group	HMG-CoA Reductase	
	Specific activity*	Protein mass**
NLP-C	8.20 ± 0.32	246 ± 17
HLP-C	1.90 ± 0.15	102 ± 6
HLP-UC	5.91 ± 0.25	173 ± 12
HLP-T3	3.85 ± 0.19 (-34.8%)‡	138 ± 6 (-20.2%)†
HLP-T4	2.81 ± 0.21 (-52.4%)‡	94 ± 5 (-45.7%)‡

*Values are mean (nmoles of mevalonate formed/min/mg protein) ± SD from three separate assay in each group.

**Values are mean (μg/g of microsomal protein) ± SD from three separate assay in each group.

NLP-C, normal control; HLP-C, hyperlipidemic control; HLP-UC, saline-fed control; HLP-T3 & HLP-T4, given 1.17 mg and 2.34 mg of purified TRF/kg/day, respectively, for 7 days.

Significantly different from HLP-UC at ‡$p<0.001$, †$p<0.01$ & ¶$p<0.05$.

diet. However, administration of purified TRF to these hyperlipidemic rats for 7 days resulted in a further decrease in enzyme activity as well as protein mass of HMG-CoA reductase. HMG-CoA reductase activity remained decreased by 35% and 52% in HLP-T3 and HLP-T4, respectively, whereas protein mass was significantly reduced by 20% and 46% as compared to saline-fed hyperlipidemic control (Table 38). These results show that purified TRF mediate its action via decrease in HMG-CoA reductase protein mass which in turn results in decreased enzyme activity and cholesterol production.

3.11.4 Effect on microsomal lipid peroxidation and plasma LDL oxidation

As shown in Table 39, formation of TBARS in microsomes was increased from 2.41 units/mg in NLP-C to 4.14 units/mg in HLP-C, whereas LDL oxidation in plasma increased from 5.31 units/ml in NLP-C to 10.17 units/ml in HLP-C, after feeding atherogenic diet for three weeks. These values were reduced to 2.81 units/mg and 7.78 units/ml in HLP-UC after 7 days of the withdrawal of atherogenic diet, but the decrease was enhanced significantly in purified TRF treated hyperlipidemic rats for 7 days. Lipid peroxidation in microsomes decreased by 29% and 43% in HLP-T3 and HLP-T4, respectively, whereas formation of conjugated dienes in plasma was reduced by 33% and 46%, respectively, (Table 35). These results demonstrate that the induction of hyperlipidemia in rats is associated with a significant increase in the microsomal lipid peroxidation as well as plasma LDL oxidation, which is significantly prevented by the administration of purified TRF.

The combined results demonstrate that hypolipidemic efficiency of purified TRF from raw Basmati (at 1.67 and 3.35 mg/kg/day) and raw Saket-4 (at 1.17 and 2.34 mg/kg/day) was comparable with the anticholesterol impact of TRF from refined RBO (at 4 and 8 mg/kg/day). These results also indicate that differential

TABLE 39

DOSE-DEPENDENT IMPACT OF PURIFIED TRF ISOLATED FROM SAKET-4 ON MICROSOMAL LIPID PEROXIDATION AND PLASMA LDL OXIDATION IN HYPERLIPIDEMIC RATS TREATED FOR 7 DAYS

Group	TBARS*	Conjugated Diene**
NLP-C	2.41 ± 0.15	5.31 ± 0.40
HLP-C	4.14 ± 0.23	10.17 ± 0.58
HLP-UC	2.81 ± 0.18	7.78 ± 0.43
HLP-T3	1.99 ± 0.12 (-29.2%)†	5.22 ± 0.42 (-32.5%)‡
HLP-T4	1.60 ± 0.13 (-43.1%)‡	4.23 ± 0.39 (-45.6%)‡

*Values are mean (nmole/min/mg) ± SD of 4 separate assay in each group.
**Values are mean (nmole/min/ml) ± SD of 3 separate assay in each group.
NLP-C, normal control; HLP-C, hyperlipidemic control; HLP-UC, saline-fed control; HLP-T3 & HLP-T4, given 1.17 mg and 2.34 mg of purified TRF/kg/day, respectively, for 7 days.
Significantly different from HLP-UC at $^{\ddagger}p<0.001$ and $^{\dagger}p<0.01$.

hypolipidemic impacts of TRF on cholesterol dynamics is directly related to the total content of γ- and δ-T_3 present in TRF isolated from a given cultivar of rice.

Based on the above results and the presence of γ- and δ-T_3 content in a given cultivar of rice, one can calculate the optimal dose of TRF in terms of its maximal cholesterol lowering efficiency. For example, as shown in Table 31, the combined content of γ- and δ-T_3 in parboiled Mansuri is 16.2%. Based on this value, the equivalent doses at 3 and 6 mg TRF/kg/day will be 2.30 and 4.60 mg purified TRF/kg/day, respectively. Similarly, an optimal dose of TRF in terms of its cholesterol lowering efficiency, equivalent to 8 mg TRF/kg/day, will be 6.12 mg purified TRF/kg/day.

The combined results indicate that variety of edible oils lacking in Tocotrienols can be supplemented with an optimal dose of TRF for its therapeutic benefits. Based on strong hypolipidemic action of TRF in normolipidemic and hyperlipidemic rats, daily intake of TRF, RBO or edible oils supplemented with TRF by normal population will prevent the occurrence of hypercholesterolemia and cardiovascular disease.

3.12 Impact of TRF on Plasma Cholesterol and Triglycerides VLDL-C, LDL-C, HDL-C, HDL_2-C, HDL_3-C, ApoB and ApoA-1 and on the Ratios of LDL-C/HDL-C, HDL-C/TC and ApoB/ApoA-1 in a Familial Hypercholesterolemic Patient With Severe Xanthomas After Different Duration of Treatment

3.12.1 Effect on plasma lipids

As shown in Table 40, the plasma total cholesterol (TC) concentrations were significantly decreased by 19%, 12%, 9% and 7% after 8, 20, 60 and 100 weeks of TRF (**8 mg/kg/day**) treatment, respectively, when compared to zero-time (entry point of the study) value. The plasma triglycerides level were decreased by 20%, 15%, 18% and 16% after 8, 20, 60 and 100 weeks of TRF treatment, respectively. These results indicate that TRF-mediated decrease of total

TABLE 40

IMPACT OF TOCOTRIENOL RICH FRACTION ON PLASMA TOTAL CHOLESTEROL AND TRIGLYCERIDES IN A FAMILIAL HYPERCHOLESTEROLEMIC PATIENT WITH SEVERE XANTHOMAS AFTER DIFFERENT DURATION OF TREATMENT

Parameters	0 Time	8 Weeks	20 Weeks	60 Weeks	100 Weeks
Total Cholesterol	440 ± 22	354 ± 18 (-19.5%)[†]	388 ± 13 (-11.8%)[¶]	402 ± 16 (-8.6%)[¶]	409 ± 17 (-7.0%)
Triglycerides	211 ± 13	169 ± 11 (-20.0%)[†]	180 ± 15 (-14.7%)[¶]	173 ± 12 (-18.0%)[¶]	178 ± 16 (-15.6%)[¶]

*Values are expressed as mean (mg/dl) ± SD from three different determinations.
Significantly different from zero-time at [†]$p<0.01$ & [¶]$p<0.05$.

cholesterol and triglycerides was higher during initial phase of TRF treatment i.e., after 8 and 20 weeks. The results also indicate that the decline in triglycerides was more marked even at 60 and 100 weeks of TRF treatment than the decrease in TC levels.

3.12.2 Impact on plasma lipoproteins lipids, apoB and apoA-1

As seen in Table 41 plasma LDL-cholesterol levels decreased by 25%, 23%, 16% and 14% after 8, 20, 60 and 100 weeks of TRF treatment, respectively, with a concomitant decrease in apoB levels, by 24%, 20%, 16% and 15%, respectively. VLDL-C levels decreased by 44%, after 8 weeks and increased by 31%, after 20 weeks but again decreased by 10% and 25% after 60 and 100 weeks of TRF treatment, respectively. Whereas, HDL-C showed an increase of 39%, 232%, 151% and 158% after 8, 20, 60 and 100 weeks of TRF treatment. ApoA-1 levels were increased by 39%, 190%, 164% and 166%, respectively, during the same period of TRF treatment. The HDL sub-fractions, HDL_2-C was substantially increased by 170%, 284%, 598% and 608%, respectively, whereas, HDL_3-C was moderately increased by 14%, 213%, 55% and 68%, respectively, after 8, 20, 60 and 100 weeks of TRF treatment, (Table 41). These results indicate that LDL-C and its apoprotein apoB, which are directly correlated with premature coronary artery disease (CAD) decreased after the treatment with TRF. The decline in LDL-C was more pronounced during initial phase of treatment. The result also indicate that the levels of HDL-C and its sub-fractions HDL_2-C and HDL_3-C which are inversely correlated to the incidence of CAD, were significantly increased due to TRF treatment.

3.12.3 Impact on the ratios of LDL-C/HDL-C, HDL-C/TC and ApoB/ApoA-1

As seen in Table 42, the LDL-C/HDL-C ratio declined by 46%, 77%, 65% and 67%, after 8, 20, 60 and 100 weeks of TRF treatment, respectively, as compared to zero-time value. Similar to LDL-C/HDL-C ratio, ApoB/ApoA-1 ratio

TABLE 41

IMPACT OF TRF ON PLASMA VLDL-C, LDL-C, HDL-C, HDL_2-C, HDL_3-C, ApoB AND ApoA-1 IN A FAMILIAL HYPERCHOLESTEROLEMIC PATIENT WITH SEVERE XANTHOMAS AFTER DIFFERENT DURATION OF TREATMENT

Parameters	0 Time	8 Weeks	20 Weeks	60 Weeks	100 Weeks
VLDL-Cholesterol	27.1 ± 2.4	15.1 ± 1.3 (-44.3%)‡	35.5 ± 3.3 (+31.0%)¶	24.5 ± 2.1 (-9.6%)	34.0 ± 2.8 (+25.4)¶
LDL-Cholesterol	392 ± 22	296 ± 11 (-24.5%)†	301 ± 13 (-23.2%)†	331 ± 17 (-15.6%)†	336 ± 15 (-14.3%)¶
HDL-Cholesterol	15.6 ± 1.5	21.7 ± 1.7 (+39.1%)†	51.5 ± 4.3 (+230.1%)‡	38.0 ± 3.2 (+143.6%)‡	39.8 ± 3.1 (+155.1%)‡
HDL_2-Cholesterol	2.5 ± 0.2	6.9 ± 0.5 (+176.0%)‡	9.8 ± 0.7 (+292.0%)‡	17.8 ± 1.2 (+612.0%)‡	18.0 ± 1.1 (+620.0%)‡
HDL_3-Cholesterol	13.0 ± 0.9	14.8 ± 0.8 (+13.8%)¶	40.6 ± 3.1 (+208.5%)‡	20.2 ± 1.6 (+55.4%)‡	21.9 ± 1.5 (+68.5%)‡
ApoB	207 ± 12	156 ± 7 (-24.6%)†	166 ± 9 (-19.8%)†	172 ± 10 (-16.9%)†	175 ± 9 (-15.4%)¶
ApoA-I	28.3 ± 1.2	39.3 ± 3.2 (+38.9%)†	82.1 ± 5.7 (+190.0%)‡	74.8 ± 4.8 (+164.3%)‡	75.4 ± 4.1 (+166.4%)‡

*Values are expressed as mean (mg/dl) ± SD from three different determinations.
Significantly different from zero-time at $^{\ddagger}p<0.001$, $^{\dagger}p<0.01$ & $^{\P}p<0.05$.

TABLE 42

IMPACT OF TRF ON THE RATIOS OF LDL-C/HDL-C, HDL-C/TC AND ApoB/ApoA-1 IN A FAMILIAL HYPERCHOLESTERLEMIC PATIENT WITH SEVERE XANTHOMAS AFTER DIFFERENT DURATION OF TREATMENT

Parameters	0 Time	8 Weeks	20 Weeks	60 Weeks	100 Weeks
LDL/HDL	25.2 ± 1.72*	13.7 ± 0.73 (-45.6%)‡	5.8 ± 0.28 (-77.0%)‡	8.7 ± 0.45 (-65.5%)‡	8.4 ± 0.37 (66.7%)‡
HDL/TC (%)	3.5 ± 0.13	6.1 ± 0.34 (+74.3%)‡	13.2 ± 0.51 (+277.1%)‡	9.4 ± 0.43 (+168.6%)‡	9.7 ± 0.48 (+177.1%)‡
ApoB/ApoA-1	7.3 ± 0.36	3.9 ± 0.15 (-46.7%)‡	2.0 ± 0.04 (-72.6%)‡	2.3 ± 0.05 (-68.5%)‡	2.3 ± 0.04 (-68.5%)‡

*Values are expressed as mean ± SD from three different determinations.
Significantly different from zero-time at ‡$p<0.001$

Normal Values : LDL/HDL ratio is 3.2 to 3.6 or below, HDL/TC (%) ratio is 20% or above

decreased by 47%, 73%, 69% and 69%, respectively at the above duration of TRF treatment. The HDL/TC ratio increased by 73%, 274%, 166% and 176% after 8, 20, 60 and 100 weeks of TRF treatment, respectively, when compared to the values at the entry point of the study. These results indicate that LDL-C/HDL-C ratio and the ratio of their apoproteins, ApoB/ApoA-1, which are considered as a better predictor for CAD risks, were significantly reduced from an initial 7.5 fold increase to a 2.5 fold increase after TRF treatment. The HDL/TC ratio was increased from a low 3.5% to 9.7% after 100 weeks of TRF treatment when compared to a normal value of 20% and above.

3.12.4 Regression of xanthomas, localized over the buttocks extending to thighs, elbows and knee after 20 weeks of TRF treatment of a familial hypercholesterolemic (FH) boy

Fig. 3.1 (Panel A and B), depicts the skin xanthomas on his buttocks extending to thighs before (entry point, Panel A) and after 20 weeks of TRF treatment (8 mg/kg/day) (Panel B) of the FH patient. It is evident that TRF treatment for 20 weeks caused a significant regression of xanthomas on his buttocks extending to thighs. Fig. 3.2 (Panel A and B) shows the stasus of skin xanthomas on left elbow before (entry point, Panel A) and after 20 weeks of TRF treatment (Panel B). Consistent with strong hypolipidemic property of TRF, a significant regression of xanthomas on patient's left elbow was observed following TRF treatment. As seen in Fig. 3.3 (Panel A and B), 20 weeks of TRF treatment of the FH patient was associated with a significant regression of xanthomas on his right elbow (Panel B), when compared to the severity of xanthomas at the entry point of the treatment (Panel A). Fig. 3.4 (Panel A and B) depicts the impact of 20 weeks of TRF treatment of the FH patient, which is associated with a significant regression of both the xanthomas located on his knee (Panel B), in comparison to the size of xanthomas before the TRF treatment (Panel A). The combined results demonstrate that the regression of cholesterol deposition in tissues at the above

Fig. 3.1 Panel A. Photograph showing skin xanthomas on buttocks, extending to thighs of FH patient at the entry point, before initiation of TRF treatment.

Panel B. Photograph showing regression of skin xanthomas after 20 weeks of TRF treatment.

Panel A

Panel B

Fig. 3.2 Panel A. **Photograph depicting skin xanthomas on left elbow of FH patient at the entry point, before initiation of TRF treatment.**

Panel B. **Photograph depicting regression of skin xanthomas after 20 weeks of TRF treatment.**

Panel A

Panel B

Fig. 3.3 Panel A. Photograph showing skin xanthomas on right elbow of FH patient at the entry point, before initiation of TRF treatment.

Panel B. Photograph showing regression of skin xanthomas after 20 weeks of TRF treatment.

Panel A

Panel B

Fig. 3.4 Panel A. Photograph depicting skin xanthomas on the knee of FH patient at the entry point, before initiation of TRF treatment.

Panel B. Photograph depicting regression of skin xanthomas after 20 weeks of TRF treatment.

Panel A

Panel B

three locations, is apparently due to a reduction in plasma triglycerides, total cholesterol, LDL-C and a concomitant increase in HDL levels, after 20 weeks of TRF treatment.

3.13 Impact of Rice Bran Oil on Plasma Cholesterol, VLDL-C, LDL-C and HDL-C and on the Ratios of LDL-C/HDL-C, HDL-C/TC in Normolipidemic Humans After Two and Four Weeks of Intake

3.13.1 Impact on Plasma Cholesterol, VLDL-C, LDL-C and HDL-C

As seen in Table 43, intake of refined edible grade RBO (**0.2 g/kg/day**), rich in tocotrienols for 2 weeks caused an insignificant decline (4%) in plasma total cholesterol level, whereas a significant decline of 10% in total cholesterol level was observed after 4 weeks of RBO intake. LDL-C levels decreased significantly by 13% and 26% after 2 and 4 weeks of RBO intake, respectively. Whereas, after 2 and 4 weeks of RBO intake HDL-C was significantly increased by 21 and 41%, respectively. VLDL-C levels after 2 and 4 weeks of RBO intake were not affected. The results demonstrate that intake of RBO equivalent to 8 mg TRF/kg/day by normolipidemic subjects caused a significant decline in lipid parameters, indicating a role of RBO in the prevention of atherogenesis.

3.13.2 Impact on the Ratios of LDL-C/HDL-C, HDL-C/TC

As shown in Table 44, LDL-C/HDL-C ratio was significantly declined by 47% after 4 weeks of RBO intake. HDL-C/TC ratio was significantly increased by 27% and 55% after 2 and 4 weeks of RBO intake, respectively. These results indicate that a 4 week intake of RBO by normal humans caused a significant improvement in the already normal ratios of LDL-C/HDL-C and HDL-C/TC, which are known to be associtaed with the development of CAD.

TABLE 43

IMPACT OF RICE BRAN OIL (RBO) ON PLASMA TOTAL CHOLESTEROL AND LIPOPROTEIN LIPIDS IN NORMOLIPIDEMIC HUMANS AFTER 2 AND 4 WEEKS OF INTAKE

Parameters	0 Time	2 Weeks	4 Weeks
Total cholesterol	197.6 ± 22.1*	189.1 ± 18.2 (-4.3%)	178.8 ± 16.8 (-9.5%)‡
LDL-cholesterol	129.5 ± 23.7	113.3 ± 14.9 (-12.5%)¶	95.8 ± 8.2 (-26.0%)†
HDL-cholesterol	36.4 ± 6.7	44.2 ± 8.0 (+21.4%)‡	51.3 ± 9.3 (+41.0%)‡
VLDL-cholesterol	25.2 ± 4.9	26.6 ± 5.4 (+5.6%)	27.3 ± 7.8 (+8.3%)

*Values are expressed as mean ± SD from three different determinations.
Significantly different from zero-time at ‡$p<0.001$, †$p<0.01$ & ¶$p<0.05$.

TABLE 44

IMPACT OF RICE BRAN OIL (RBO) ON THE RATIOS OF LDL-C/HDL-C, HDL-C/TC IN NORMOLIPIDEMIC HUMANS AFTER 2 AND 4 WEEKS OF INTAKE

Parameters	0 Time	2 Weeks	4 Weeks
LDL-C/HDL-C	3.62 ± 0.66*	2.59 ± 0.31 (-28.2%)‡	1.93 ± 0.19 (-46.5%)‡
HDL/TC%	18.4 ± 0.9	23.3 ± 1.3 (+26.9%)‡	28.5 ± 1.7 (+55.4%)‡

*Values are expressed as mean ± SD from three different determinations.
Significantly different from zero-time at ‡$p<0.001$
Normal Values : LDL/HDL ratio is 3.2 to 3.6 or below, HDL/TC% ratio is 20% or above

Discussion

The results presented in the thesis describe a rapid, efficient, and cost-effective method for the isolation of tocotrienol and tocopherol rich fraction (TRF) from rice bran, crude rice bran oil (RBO) or refined edible grade RBO. Previously reported methods for the isolation of TRF involve time consuming multiple steps, including saponification of the oil (Sharma and Rukmini, 1987; Shin and Godber, 1994). Our method simply involves the extraction of TRF from rice bran/RBO in methanol (Beg *et al.*, 1996a). Precaution is taken that within 24 h of milling, brans from raw paddies are soaked in hexane. This is essential because during milling, lipase enzyme is activated which hydrolyzes triglycerides to free fatty acids (Migura, 1989). Free fatty acid rise in raw bran is very rapid, 6-10% in 24-48 h of milling, whereas only 2-3% in 48 h in case of parboiled bran, where lipase enzyme is apparently partially inactivated by parboiling. The yield of TRF isolated from refined edible grade RBO is 4.3%, which is consistent with the values reported earlier (Sharma and Rukmini, 1987). However, the percent yield of TRF isolated from crude RBO from brans of raw and parboiled Basmati and Saket-4 cultivars varied between 6.6 and 7.2. The variation in the percent yield of TRF is indicative of the fact that types, α-, β-, γ- and δ-tocotrienols (T_3) and tocopherols (T) and their quantity may vary in the raw and parboiled brans representative of two cultivars of rice. The TRF content of refined edible grade RBO isolated by the above methodology was significantly lower, 4.3%, suggesting the possibility of some removal of T_3/T during refining procedures of the commercial edible grade RBO. The TRF, thus obtained, is purified involving a single step purification procedure. The yield of purified TRF from TRF of crude RBO from raw and parboiled cultivars, was in the range of 4.3-4.9%, which is on an average ~ 62% higher than ~ 2.8% yield of purified TRF obtained from TRF of refined edible grade RBO. The combined results demonstrate that the isolation of purified TRF from crude RBO by employing the above methods yields a significantly higher quantity of value-added products, tocotrienols and tocopherols, in comparison to

purified TRF isolated from refined edible grade RBO. In addition, the cost involved in the isolation of TRF from crude RBO derived from brans of different cultivars of rice is far less than the cost involved in the isolation of TRF from commercially procured refined edible grade RBO.

The combined content of α-, β-, γ- and δ-tocotrienols and tocopherols, as quantified by HPLC, in TRF isolated from refined edible grade RBO was 29.2% and 39.1%, respectively, whereas, for purified TRF the total content of T_3 and T was 42.2% and 57.9%. The increase in percent content of the combined T_3 and T in purified TRF was consistent with ~35% of the non-T_3 and T contaminants removed during the purification of TRF. Therefore, increase in combined percent yield of T_3 and T in purified TRF of two cultivars of rice was in agreement with the ~35% contaminants associated with TRF. Since, it is known that γ and δ-T_3, are most potent in terms of their *in vivo* hypolipidemic, (Pearce *et al.*, 1992; Qureshi and Qureshi, 1993), *ex vivo* and *in vitro* antioxidant, (Suarna *et al.*, 1993; Kamat and Devasagayam, 1995; Kamat *et al.*, 1997) and *in vivo* and *in vitro* anti-tumour (Goh *et al.*, 1994; Nesaretnam *et al.*, 1995; Guthrie *et al.*, 1997; Nesaretnam *et al.*, 1998) activities, the combined percent content of δ- and γ-T_3 present in purified TRF of Basmati and Saket-4 was significantly different than that of purified TRF obtained from refined edible grade RBO.

A detailed investigation of TRF and purified TRF, as isolated above, on cholesterol dynamics of normolipidemic and hyperlipidemic rats has been carried out. Feeding of tocotrienol as TRF, isolated from refined edible grade RBO to normal rats for two weeks was associated with a significant decline in plasma and lipoprotein lipids. It is interesting to note that both TRF and purified TRF mediated a significant decline in total and esterified cholesterol levels, with no significant change in free cholesterol. The results demonstrate that decrease in

plasma total cholesterol level is apparently due to a significant decrease in VLDL-C and LDL-C levels. Administration of TRF or purified TRF caused a significant decline in plasma apolipoprotein B associated with LDL. Although no significant change in HDL-C was observed, but its sub-fraction, HDL_2-C, which is considered to be a strong predictor of presence and extent of coronary artery disease (CAD) (Drexel *et al.*, 1992), was significantly increased. Consistent with no change in HDL-C, its apolipoprotein A-1 level was not affected.

In order to find out the minimum dose to exert a maximal hypolipidemic effect, we investigated the impacts of 4, 8 and 12 mg TRF/kg/day and 2.6, 5.2 and 7.8 mg purified TRF/kg/day on cholesterol dynamics in normolipidemic rats for 3 weeks. The combined results indicate that administration of TRF and purified TRF to normolipidemic rats was associated with a significant decline in plasma levels of triglycerides, total cholesterol, LDL-C and apoB in a dose-dependent manner. No effect on HDL-C and apoA-1 was observed, whereas, HDL sub-fraction, HDL_2-C was significantly increased with a decline in HDL_3-C. In general, our results are consistent with several scattered reports indicating hypolipidemic action of tocotrienols, in normolipidemic animal and human models, administered as RBO, palm oil, barley oil or TRF isolated from either RBO or palm oil. Feeding of a diet containing 5-20% RBO to normal rats was associated with a significant decline in serum total cholesterol, LDL-C and VLDL-C (Sharma and Rukmini, 1986; Purushothama *et al.*, 1995), whereas triglycerides and HDL-C levels were not significantly changed (Sharma and Rukmini, 1986). Earlier findings from our laboratory (Beg *et al.*, 1996b) related to hypolipidemic action of TRF in normolipidemic humans are consistent with the results reported in the thesis. Our results are also consistent with a decline in plasma cholesterol in hamsters fed 9% RBO for 3 weeks (Kahlon *et al.*, 1992). Diet containing 35% RBO when fed to normolipidemic monkeys also resulted in a

significant decline in total cholesterol, LDL-C and apoB, with no change in HDL-C (Nicolosi *et al.*, 1991). Feeding of TRF (50 μg/g of diet) for 6 and 8 weeks to normolipidemic swines was associated with a significant decline in total cholesterol, LDL-C and apoB with no change in HDL-C and apoA-1 levels (Qureshi *et al.*, 1991c). Consistent with the hypocholesterolemic action of TRF in rats reported here, Pearce *et al.* (1992) have demonstrated a significant hypolipidemic effect of α-, γ- and δ-T_3 in normolipidemic chickens. Feeding of α-, γ- and δ-T_3 (20 μg/g of diet) to normolipidemic chickens for 4 weeks resulted in the significant decline in serum total cholesterol and LDL-C levels with no change in HDL-C level. As expected, hypolipidemic impact of α-T_3 was lower than γ- and δ-T_3 (Qureshi and Qureshi, 1993). Feeding of dietary palm oil (15-20%) to normolipidemic rats resulted in varied effects on triglycerides, total cholesterol, LDL-C, HDL-C and HDL-C/total cholesterol ratio (Pereira *et al.*, 1990; Sundram *et al.*, 1990; Osim *et al.*, 1996). Similar results were observed when 70% dietary palm oil was fed to normolipidemic humans (Sundram *et al.*, 1992). However, feeding of one capsule/day of 18 mg tocopherols and 42 mg tocotrienols in 240 mg of palmolein for 30 days to normolipidemic humans, resulted in a significant decline in total cholesterol and LDL-C with inconsistent change in triglycerides and HDL-C levels (Tan *et al.*, 1991). Similarly, feeding of barley oil to normolipidemic chickens significantly reduced the levels of plasma total cholesterol and LDL-C with no change in triglycerides and HDL-C levels (Qureshi *et al.*, 1986).

Next, we investigated the role of purified TRF in the prevention of experimental hyperlipidemia in rats. In addition, the efficacy of purified TRF as an anticholesterol drug in the treatment of experimental hyperlipidemia was also investigated. Feeding of 65 mg purified TRF/kg/day along with an atherogenic diet to rats for 3 weeks, significantly prevented the increase in triglycerides, total cholesterol including esterified and free cholesterol, VLDL-C, LDL-C and apoB

concentrations when compared to rats fed only atherogenic diet. Increase in apoA-1, HDL-C and its sub-fraction HDL_3-C levels, observed apparently due to feeding of an atherogenic diet, significantly decreased due to supplementation of purified TRF. However, no preventive action of TRF was seen in HDL_2-C level. Administration of 65 mg purified TRF/kg/day to normolipidemic rats for 3 weeks exerted hypolipidemic effects consistent with the data discussed above, where 81.25 mg purified TRF/kg/day was fed to normal rats for 2 weeks. The results demonstrate that 5 and 7 days after the withdrawal of atherogenic diet, plasma triglycerides, total cholesterol including esterified and free cholesterol, VLDL-C, LDL-C including apoB, HDL-C and its sub-fraction, HDL_2-C and HDL_3-C levels were significantly reduced. Apo A-1 level was reduced insignificantly. Feeding of 16.25 and 32.50 mg of purified TRF/kg/day to hyperlipidemic rats for 5 and 7 days resulted in further significant reduction in the above mentioned plasma and lipoprotein lipids including apoB in comparison to untreated hyperlipidemic control. The significant decline in total cholesterol level mediated by TRF is due to a significant decrease in VLDL-C and LDL-C levels. The combined results demonstrate that hypolipidemic impacts at a dose of 16.25 and 32.50 mg purified TRF/kg/day for 5 and 7 days were similar, indicating that an optimal dose of purified TRF may be significantly lower than 16.25 mg/kg/day.

In order to find out the minimum dose which can exert maximal hypolipidemic effect, we also investigated the impacts of 2.6, 5.2 and 7.8 mg purified TRF/kg/day on cholesterol dynamics in hyperlipidemic rats for 7 days. Administration of 2.6, 5.2 and 7.8 mg purified TRF/kg/day to hyperlipidemic rats for 7 days was associated with a dose-dependent decline in plasma triglycerides, total cholesterol including esterified and free cholesterol, VLDL-C, LDL-C and apoB. No affect on HDL-C and HDL_3-C levels was observed at the 3 doses of purified TRF, whereas, slight change in the levels of HDL_2-C and apoA-1 was

seen. The combined results demonstrate that a maximal hypolipidemic impact of purified TRF is exerted at a dose of 5.2 mg/kg/day in hyperlipidemic rats. These results also support the contention that a maximal efficacy of purified TRF in terms of its cholesterol lowering properties is obtained at 5.2 mg/kg/day which is similar to an optimal dose of 5.2 mg purified TRF/kg/day or 8 mg TRF/kg/day obtained in normolipidemic rats.

Our results are supported by earlier studies where RBO (10%), TRF (0.2-0.4%) or γ-T_3 (50 μg/g of diet) were fed to rats along with cholesterol-rich diet for 6-8 weeks, which prevented significantly the increase in total cholesterol, LDL-C and VLDL-C. However, TRF did not affect significantly the HDL-C and triglycerides levels (Sharma and Rukmini, 1986 & 1987; Seetharamaiah and Chandrasekhara, 1989; Watkins *et al.*, 1993). Similarly, feeding of tocotrienols together with atherogenic diet to rabbits for 12 weeks prevented significantly increase in serum total cholesterol and LDL-C (Teoh *et al.*, 1994). Analogous to hypocholesterolemic impacts of TRF in experimental hyperlipidemic rats and rabbits, a significant decline in total cholesterol, LDL-C and apoB was observed in pigs, with inherited hyperlipidemia, fed TRF (50 μg/g of diet), isolated from palm oil, for 6 weeks. Similar to our results in rats, no affect on HDL-C and apoA-1 was observed in genetically hyperlipidemic pigs treated with TRF (Qureshi *et al.*, 1991c). Feeding of a diet supplemented with purified α-, γ- and δ-T_3 from palm oil (20 μg/g of diet) to hypercholesterolemic chickens significantly reduced serum total cholesterol and LDL-C levels. Feeding of one capsule/day of 18 mg tocopherols and 42 mg tocotrienols in 240 mg of palmolein for 30 days to hyperlipidemic humans, resulted in a significant decline in total cholesterol and LDL-C with inconsistent change in triglycerides and HDL-C levels (Tan *et al.*, 1991). Consistent with our results, TRF/T_3 exhibited more potent hypocholesterolemic action in inherited hyperlipidemic swines and experimental

hypercholesterolemic chickens comparison to normolipidemic swines and chickens (Qureshi *et al.*, 1991c; Qureshi and Qureshi, 1993). The hypolipidemic effects of dietary RBO, TRF or γ-T_3 in hyperlipidemic humans were consistent with our results observed in hyperlipidemic rats, and reported by others in rat (Sharma and Rukmini, 1986 & 1987; Seetharamaiah and Chandrasekhara, 1989; Watkins *et al.*, 1993), rabbit (Teoh *et al.*, 1994), pig (Qureshi *et al.*, 1991c) and avian (Qureshi and Qureshi, 1993) models. Initial findings from our laboratory related to hypolipidemic effect of dietary RBO/TRF in hyperlipidemic rats (Beg *et al.*, 1996b) and humans (Khan *et al.*, 1994; Beg *et al.*, 1995 & 1996b) and repored by others in humans (Tan *et al.*, 1991) are consistent with the results reported in the thesis and published, in part, elsewhere (Beg *et al.*, 1997; Minhajuddin *et al.*, 1999). Intake of dietary RBO as cooking oil for 2 and 4 weeks by hypercholesterolemic humans resulted in a significant decrease of plasma triglycerides and total cholesterol levels (Raghuram *et al.*, 1989). In another study, feeding of 200 mg TRF/day from palm oil or 200 mg γ-T_3/day for 4 weeks to hypercholesterolemic humans was associated with a significant decrease in total cholesterol, LDL-C and apoB levels, whereas, triglycerides, HDL-C and apoA-1 levels were not affected (Qureshi *et al.*, 1991a). Treatment of patients with hyperlipidemia and carotid stenosis with TRF (240-360 mg/day) isolated from palm oil for 12 months revealed a significant apparent carotid atherosclerotic regression with no change in serum triglycerides, total cholesterol, LDL-C and HDL-C (Tomeo *et al.*, 1995).

The hypocholesterolemic action of TRF and purified TRF in normolipidemic and hyperlipidemic rats is mediated by a decrease in the enzymatic activity of HMG-CoA reductase, the rate-limiting enzyme in the biosynthetic pathway of cholesterol. A concomitant decline in immuno-reactive HMG-CoA reductase protein mass appears to be the reason for a decrease in enzyme activity and a

consequent decline in cholesterol production. Administration of TRF (125 mg/kg/day) and purified TRF (81.25 mg/kg/day) for 2 weeks to normolipidemic rats was associated with a significant decrease in the enzymatic activity of HMG-CoA reductase and its protein mass. Similar results were obtained in normolipidemic rats treated with 4, 8 and 12 mg TRF/kg/day or 2.6, 5.2 and 7.8 mg purified TRF/kg/day for 3 weeks in a dose-dependent manner. Feeding of cholesterol rich (atherogenic) diet to rats for 3 weeks resulted in a sharp decrease in the enzymatic activity and protein mass of HMG-CoA reductase. These results are consistent with the well known negative feedback inhibition of HMG-CoA reductase mediated by feeding of high levels of cholesterol. Supplementation of purified TRF (65 mg/kg/day) with the atherogenic diet for 3 weeks further reduced the enzymatic activity and protein mass of HMG-CoA reductase, indicating the possibility of a different mechanism of cholesterol regulation than cholesterol itself. Five and seven days after the withdrawal of atherogenic diet in untreated hyperlipidemic rats, the enzymatic activity and protein mass of HMG-CoA reductase was significantly increased due to release of cholesterol feedback inhibition. However, the decline in total cholesterol and LDL-C levels by administering several doses of TRF or purified TRF for 5 and 7 days to hyperlipidemic rats is apparently mediated by a significant reduction in enzymatic activity of HMG-CoA reductase, which in turn is reduced by a decrease in its protein mass. Consistent with maximal hypolipidemic impacts of TRF, an optimal reduction in the enzymatic activity and protein mass of HMG-CoA reductase was obtained at 8 mg TRF/kg/day or 5.2 mg purified TRF/kg/day. The combined data provide sufficient evidence that tocotrienols present in TRF exert their hypolipidemic effects in normolipidemic as well as hyperlipidemic rats by a common mechanism involving the reduction in HMG-CoA reductase activity, which in turn is reduced by a decline in its protein mass.

The combined results presented here indicate that tocotrienols are highly effective in lowering blood cholesterol and LDL-C levels by repressing HMG-CoA reductase. It has been established that the suppression of HMG-CoA reductase requires two regulators, cholesterol delivered by receptor-mediated uptake of LDL and non-sterol product derived from mevalonate. The former is expressed predominantly through the changes in the rate of transcription of the HMG-CoA reductase gene, and the latter by modulating the efficiency of translation of HMG-CoA reductase mRNA or by the degradation of protein (Goldstein and Brown, 1990). The exact mechanism by which tocotrienols act *in vivo* at the cellular level has not been established. However, in normolipidemic and hyperlipidemic chickens, TRF/T_3 have been shown to suppress the hepatic HMG-CoA reductase activity (Pearce *et al.*, 1992; Qureshi and Qureshi, 1993), whereas, enzymatic activity of HMG-CoA reductase in adipose tissue of normolipidemic and hypercholesterolemic swines was reduced after TRF treatment (Qureshi *et al.*, 1991a). Our results in normolipidemic and hyperlipidemic rats demonstrate that TRF suppresses the hepatic HMG-CoA reductase activity by reducing its protein mass. These studies represent an initial demonstration of the TRF mediated *in vivo* mechanism of suppression of HMG-CoA reductase activity involving a concomitant reduction in the protein mass. The decline in protein mass may be achieved by inhibition of HMG-CoA reductase synthesis and/or enhanced degradation. Similar to our *in vivo* results, γ-T_3 has been shown to mediate the suppression of enzymatic activity and protein mass of HMG-CoA reductase in HepG2 cells, through decreased synthesis (57% of control) and enhanced degradation (2.4 fold versus control) of the enzyme (Parker *et al.*, 1993). Thus, tocotrienols influence the mevalonate pathway in mammalian cells *in vitro*, by post-transcriptional suppression of HMG-CoA reductase, and appear to specifically modulate the intracellular mechanism for controlled degradation of the reductase protein (Parker *et al.*, 1993). These activities of tocotrienols in HepG2

cells mirror the actions of the putative non-sterol feedback regulators derived from mevalonate in cultured cells (Goldstein and Brown, 1990). TRF mediated *in vivo* mechanism of inhibition of enzymatic activity and its protein mass of HMG-CoA reductase in normolipidemic and hyperlipidemic rats, as demonstrated in the present study, may be analogous to the mechanism shown in HepG2 cells (Parker *et al.*, 1993). Taken together, the available information indicate an association between the suppression of hepatic HMG-CoA reductase and cholesterogenesis, and the observed plasma cholesterol lowering activity of TRF in animal models. However, elucidation of precise *in vivo* mechanism(s) of TRF mediated inhibition of HMG-CoA reductase at molecular level remains to be investigated.

A growing body of evidence has linked processes involving oxygen-derived free radicals with the initiation and propagation of atherosclerosis. In particular, the oxidative modification of LDL enhances its atherogenecity (Steinberg, 1988; Duthie *et al.*, 1989; Palinski *et al.*, 1989). Recent studies have demonstrated that oxidized lipids, especially oxidized fatty acids and cholesterol in the diet are atherogenic (Staprans *et al.*, 1998). An inadequate intake of foods containing antioxidant vitamins could result in the oxidation of LDL-C. Antioxidant, especially vitamin E (tocopherols), have recently received considerable attention as potential anti-atherogenic agents. Several studies have demonstrated the ability of tocopherols to prevent *ex vivo* and *in vitro* LDL-C oxidation and significantly reduce the development of atherosclerotic lesions (Carew *et al.*, 1987; Esterbauer *et al.*, 1991; Mao *et al.*, 1991), risk of coronary heart disease (CHD) (Rimm *et al.*, 1993; Stampfer *et al.*, 1993) and ischemic heart disease (Gey, 1995). As the TRF isolated from RBO is enriched with T_3 and T, and tocotrienols are known to be more potent antioxidant than tocopherols (Suarna *et al.*, 1993; Kamat and Devasagayam, 1995; Kamat *et al.*, 1997), we have investigated the antioxidant impacts of TRF in normolipidemic and hyperlipidemic rats.

Feeding of TRF and purified TRF to normolipidemic rats for 2 weeks was associated with a significant decline, in microsomal lipid peroxidation, quantified as thiobarbituric acids reactive substances (TBARS) and conjugated dienes of plasma LDL oxidation (conjugated dienes). Similarly, feeding of 4, 8 an 12 mg TRF/kg/day and 2.6, 5.2 and 7.8 mg purified TRF/kg/day for 3 weeks inhibited peroxidation of lipids and oxidation of LDL in a dose-dependent manner. Based on the combined results one can conclude that a dose of 8 mg TRF/kg/day or 5.2 mg purified TRF/kg/day exerts a maximal hypolipidemic and antioxidant effect in normolipidemic rats. These results are consistent with earlier reports indicating an inhibition in the formation of TBARS and conjugated dienes by TRF or individual T_3 and T when added *in vitro* in rat brain mitochondria (Kamat and Devasagayam, 1995) and in rat liver microsomes (Kamat *et al.*, 1997). Indirect support to our results was obtained from an earlier report indicating an inhibition in the formation of lipid hydroperoxides in rats and humans treated with α- and γ-T_3 (Suarna *et al.*, 1993).

Since antioxidants may have a role in the prevention of hyperlipidemia and atherosclerosis, we also investigated the antioxidant impact of TRF on microsomal lipid peroxides (TBARS) and plasma LDL oxidation (conjugated dienes) in hyperlipidemic rats. Inhibition in the formation of lipid peroxides (TBARS) and LDL oxidation (conjugated dienes) in TRF treated (65 mg purified TRF) normal rats was similar to the antioxidant activity expressed by feeding 81.25 mg purified TRF for 2 weeks as shown in Table 6. In response to oxidative stress, evoked in experimental hyperlipidemia in rats, as reflected by increased (approximately 2 fold) formation of TBARS and conjugated dienes, was substantially blocked by TRF feeding along with atherogenic diet. These results underline the importance of TRF as a potent antioxidant agent, because TRF suppressed the formation of

lipid peroxides (TBARS) and conjugated dienes during experimental hyperlipidemia, to a level lower than (24-38%) the observed values of normolipidemic rats. Treatment of hyperlipidemic rats with 16.25 and 32.50 mg purified TRF/kg/day for 5 and 7 days not only caused significant decline in lipid peroxides and conjugated dienes in comparison to hyperlipidemic untreated control rats but further reduced these levels below normal values. In agreement with the above results, treatment of hyperlipidemic rats with 2.6, 5.2 and 7.8 mg purified TRF/kg/day caused a dose-dependent decline in lipid peroxides and diene conjugates below normal values. These results demonstrate that strong hypolipidemic impacts of TRF in conjunction with potent antioxidant property can provide additional therapeutic benefit in the prevention and treatment of atherogenesis.

Our combined results demonstrating a strong antioxidant impacts of TRF (containing mixture of tocotrienols and tocopherols) in hyperlipidemic rats are in agreement with earlier reports indicating that feeding of atherogenic diet to rats, supplemented with γ-T_3 (50 μg/g of diet) for 6 weeks caused a significant prevention in the formation of plasma lipid peroxides (TBARS). However, in order to get a similar level of preventive effect in the formation of lipid peroxides (TBARS), feeding of ten fold higher concentration of α-T (500 μg/g of diet) along with atherogenic diet was required, when compared to rats fed atherogenic diet alone. These results indicate that γ-T_3 exerts a significantly more potent antioxidant impact as compared to α-T (Watkins *et al.*, 1993). Support to our results is also obtained from another study where feeding of a mixture of tocotrienols along with an atherogenic diet to rabbits was associated with a significant reduction in the formation of serum lipid peroxides (Teoh *et al.*, 1994). Treatment of patients with hyperlipidemia and carotid stenosis with TRF (240-360 mg/day), isolated from palm oil, for 8 months, caused a significant decrease in

TBARS, an *ex vivo* indicator of maximal platelet peroxidation (Tomeo *et al.*, 1995).

Vitamin E (tocopherols) is well accepted as the first line of defense against lipid peroxidation, protecting polyunsaturated fatty acids in cell membranes through its free radical quenching activity in bio-membranes at an early stage of free radical attack (Horwitt, 1986; Van Gossum *et al.*, 1988; Packer, 1991). Studies in animal and human models have indicated that plasma α-T concentrations are highly correlated with plasma total lipid levels. In addition, vitamin E is transported in the plasma solely by lipoproteins; there are no other specific plasma transport proteins (Rubinstein *et al.*, 1969; Carr *et al.*, 1993). Similar to tocopherols, tocotrienols are also transported in the blood in association with circulating lipoproteins, as has been demonstrated in rats and human models (Suarna *et al.*, 1993). Therefore, it appears that TRF exerts its antioxidant effect on plasma LDL oxidation while being attached to LDL particle.

Previously published scattered reports have shown that neither rice bran nor RBO lowered cholesterol levels. This discrepancy was explained by findings that some, but not all, rice cultivars contain tocotrienols which exert powerful hypolipidemic action (Qureshi *et al.*, 1986; Qureshi *et al.*, 1989). Because of these variation in the types and content of T_3, one finds corresponding variation in the lipid lowering affects of RBO/TRF isolated from different cultivars of rice. Out of α-, β-, γ- and δ- forms of T_3, γ and δ- T_3 have been found to be most potent in terms of their HMG-CoA reductase inhibition as well as cholesterol lowering effects. The efficiency of hypocholesterolemic action as well as the degree of inhibition of HMG-CoA reductase activity mediated by α-T_3 was substantially lower than γ and δ- T_3 (Pearce *et al.*, 1992). β-Form of T_3 failed to exhibit any anticholesterol activity. Similarly, tocopherols fail to inhibit cholesterol synthesis

at the level of rate limiting enzyme, HMG-CoA reductase in chickens, rat liver hepatocytes and HepG2 cells (Pearce *et al.*, 1992), hence do not lower cholesterol. Earlier studies in humans demonstrated that tocopherol supplementation to ones diet had no effect on serum cholesterol concentration (Isai *et al.*, 1978; Schwartz and Rutherford, 1981; Ehnholm *et al.*, 1982; Kesaniemin and Grundy, 1982; Stampfer *et al.*, 1983), or actually resulted in slight increase in serum cholesterol concentrations in some individuals (Chase *et al.*, 1980; Howard *et al.*, 1982). Similar observations were made in chickens, where administration of tocotrienols blended with increasing concentrations of tocopherols, significantly attenuated the inhibition of cholesterol biosynthesis (Qureshi *et al.*, 1996). Therefore, the hypocholesterolemic effect of TRF (containing tocotrienols and tocopherols), isolated from palm oil, is exerted through the tocotrienols (Tan *et al.*, 1991). Consistent with these reports and our results, the observed hypolipidemic impact of TRF, isolated from RBO, is apparently mediated by tocotrienols. In addition, our results indicate that the total percent content of tocopherols present in TRF is not sufficient to attenuate the anticholesterol action of tocotrienols.

Based on our findings, indicating that 8 mg TRF/kg/day or 5.2 mg purified TRF/kg/day exerts a maximal hypolipidemic effect, we investigated the hypolipidemic impacts of purified TRF isolated from two cultivars of rice, raw Basmati and Saket-4, which differ in their γ and δ- T_3 content. Based on the total content of γ and δ- T_3 present in the purified TRF of each cultivar, hypolipidemic efficiencies at a dose of 1.67 and 3.35 mg purified TRF/kg/day from Basmati and 1.17 and 2.34 mg purified TRF/kg/day from Saket-4 was investigated in hyperlipidemic rats for 7 days. The above calculated doses will be equivalent to combined concentration of γ and δ- T_3 present in 3 and 6 mg TRF from refined edible grade RBO. In addition, this calculation of TRF doses from two cultivars of rice is based on the observed maximal effect of 8 mg TRF/kg/day from refined

edible grade RBO. As expected, feeding of the above two doses of purified TRF from each cultivar to hyperlipidemic rats for 7 days was associated with a significant decline in plasma triglycerides, total cholesterol including esterified and free cholesterol, VLDL-C, LDL-C, apoB and enzymatic activity and protein mass of HMG-CoA reductase. It is important to note that at 6 mg equivalent dose of purified TRF/kg/day from both the cultivars, a twofold decrease in total cholesterol and LDL-C was observed in comparison to the hypolipidemic effect seen at 3 mg equivalent dose. In general, a dose-dependent impact of TRF on the above lipid parameters was observed. These results indicate that the hypolipidemic impacts of calculated doses from purified TRF of two rice cultivars are consistent with the observed effects seen at 4 and 8 mg TRF/kg/day. The results also demonstrate that efficacy of hypolipidemic action of TRF or purified TRF is directly related to the total content of γ and δ- T_3 (Qureshi and Qureshi, 1993). The combined results also show that the TRF lowers plasma total cholesterol and LDL-C concentrations more effectively (twofold) in hyperlipidemic rats as compared to normolipidemic rats. Based on the combined results, one can calculate an optimal dose of TRF in terms of its maximal hypolipidemic effect in a given cultivar, which is proportional to the combined quantity γ and δ- T_3 (Table 31). Consistent with earlier results, administration of 3 and 6 mg equivalent doses of purified TRF/kg/day from two cultivars of rice to hyperlipidemic rats for 7 days significantly reduced the formation of TBARS and conjugated dienes in a dose-dependent manner. Antioxidant effects of TRF coupled with its hypolipidemic property in normolipidemic and hyperlipidemic rats underline the importance of TRF in the prevention and treatment of hyperlipidemia and coronary heart disease.

The combined results presented in the thesis provide considerable evidence that tocotrienol rich fraction isolated from rice bran oil as a non saponifiable

fraction exhibit a potent hypolipidemic and antioxidant activities in normolipidemic as well as hyperlipidemic rats. Tocotrienol rich fraction used in the present investigation is rich in both tocotrienols and tocopherols. Previously published reports have suggested that tocotrienols mediate a potent hypolipidemic activity when administered in the diet to animals, including humans, (Pearce *et al.*, 1992; Qureshi *et al.*, 1995), an attribute not observed with tocopherols (Kesaniemi and Grundy, 1982; Ehnholm *et al.*, 1982). Side-chain unsaturation, which is absent in tocopherols, is important for this activity of tocotrienols. In studies, directed toward a possible mechanism of action, Qureshi *et al.* (1986) observed that the plasma cholesterol lowering effect of TRF, from barley oil, in chickens and rats, was associated with decreased levels of hepatic HMG-CoA reductase activity. Furthermore, the structure-activity relationship for the hypocholesterolemic activity of T_3 revealed that the natural and synthetic T_3 lacking the 5-methyl ring substitution, viz. γ-T_3 and δ-T_3 were highly active as suppressors of HMG-CoA reductase activity and cholesterol biosynthesis in primary rat hepatocytes and human hepatoma HepG2 cells (Pearce *et al.*, 1992). In general, our results are in agreement with the reported findings that presence of γ- and δ-T_3 in TRF is mainly responsible for the strong impact on cholesterol dynamics of normal and hyperlipidemic rats. In addition, our results demonstrate that efficacy of TRF in terms of cholesterol lowering activities varies due to the difference in the quantity of γ- and δ-T_3, which differ from cultivar to cultivar. Based on these observations, an optimal dose of 8 mg TRF/kg/day or 5.2 mg purified TRF/kg/day, fractionated from commercial refined edible grade RBO, which exerts a maximal hypolipidemic impact in normolipidemic and hyperlipidemic rats has been found. Similarly, based on the 22.2% and 31.8% combined content of γ and δ- T_3 in Basmati and Saket-4, respectively, an optimal dose of 4.47 and 3.12 mg purified TRF/kg/day was found in hyperlipidemic rats. In addition, based on the above results, and the combined content of γ- and δ-T_3 in a given cultivar, one can calculate a dose of

TRF for the maximal hypolipidemic activity of TRF. For example, an optimal dose of 6.12 mg purified TRF/kg/day has been calculated for Mansuri cultivar, based on total γ- and δ-T_3 content present in TRF (Table 31).

Our combined results demonstrate a strong hypolipidemic action of TRF, when administered to normolipidemic and hyperlipidemic rats. In particular, total cholesterol, LDL-C and apoB, which are positively associated with coronary heart disease, were significantly reduced. Levels of plasma HDL-C and apoA-1, which are considered as anti-atherogenic, were not influenced in normal and hyperlipidemic rats treated with different doses of TRF or purified TRF. However, in normolipidemic rats treated with TRF, HDL_2-C, which is considered as strong predictor of the presence and extent of CAD (Drexel et al., 1992), was significantly increased. Several epidemiological studies have demonstrated that elevated triglycerides level is associated with increased risk of CHD (Albrink and Man, 1959; Hulley *et al.*, 1980; Aberg *et al.*, 1985; Freedman *et al.*, 1988; Austin, 1991), a risk that is especially high in subjects with low HDL-cholesterol (Castelli, 1986). However, the status of triglycerides as an independent risk for CAD continues to be controversial. In our studies, TRF and purified TRF also mediated a significant decline in plasma triglycerides level of both normolipidemic and hyperlipidemic rats.

A five year old boy with severe skin xanthomas was referred to J.N. Medical College. Laboratory evaluation at entry point disclose that the child had a total cholesterol concentration of 440 mg/dl, a fasting triglycerides concentration of 211 mg/dl, LDL-C concentration of 392 mg/dl and a HDL-C concentration of 15.6 mg/dl. HDL_2-C was only 2.5 mg/dl, whereas HDL_3-C level was 13.0 mg/dl. Consistent with high LDL-C level, the apoB level was 207 mg/dl, whereas apoA-1 concentration was 28.3 mg/dl. The LDL-C/HDL-C ratio of the patient was 25.2, when ratio of normal humans is in the range of 3.2-3.6 or below. Similarly,

HDL-C/total cholesterol ratio at the entry point was 3.5% when compared to percent ratio of 20 or above in normal subjects. The child presented with classical case of familial hypercholesterolemia including a rapidly growing severe eruptive skin xanthomas on buttocks extending to thighs, less severe on elbows and couple of them on the knee. Based on above characterization the patient can be categorized as familial hypercholesterolemic. Considering the rare availability of such type of patients, the child was started on low fat, cholesterol-free diet, with 8 mg TRF/kg/day for 100 weeks. With this regimen, his triglycerides, total cholesterol and LDL-C were reduced by 20-25% after 8 weeks of TRF treatment. However, after 20 weeks, for unknown reasons, efficiency of TRF treatment in terms of its hypolipidemic action was lower than the initial phase of therapy, which was also reflected in the degree of regression of growing skin xanthomas.

Since, in humans, FH is associated with an elevated plasma total and LDL-cholesterol and its apoB levels, data from several epidemiological studies document a significant and consistent relationship between elevated levels of plasma total cholesterol and LDL-C and increased incidence of CHD (Stamler et al., 1986; Castelli et al., 1986; Martin et al., 1986) and atherosclerotic lesions (Solberg and Strong, 1983). A significant reduction in total cholesterol, LDL-C and apoB was seen in a FH patient following 100 weeks of TRF treatment. Numerous studies have also demonstrated an inverse relationship between plasma levels of HDL-C including apoA-1, CHD incidence (Goldbourt et al., 1985; Gordan et al., 1989; Goldbourt and Yaari, 1990) and coronary atherosclerosis (Solberg and Strong, 1983). Our results demonstrate a substantial increase in the levels of HDL-C (155%) and apoA-1 (166%) after 100 weeks of TRF treatment. There is a mounting evidence that lower levels of HDL-C predict CAD even when total cholesterol levels are not elevated (Brunner et al., 1987; Abbott et al., 1988; Miller et al., 1990). Case-control studies indicate that the inverse relationship with

CAD is mainly due to HDL subfraction, HDL_2-C, which is the strongest predictor of both presence and extent of CAD (Drexel *et al.*, 1992). Our results are consistent with these reported findings indicating a TRF mediated significant increase of 620% in HDL_2-C level after 100 weeks of treatment. Intervention with repeated coronary arteriographics to monitor disease progression have consistently shown that ratios of LDL-C/HDL-C, apoB/apoA-1 and HDL-C/total cholesterol are independently associated with growth of atherosclerotic lesion (Levy et al., 1984; Arntzenius et al., 1985; Nikkila et al., 1994). Consistent with these findings, 100 weeks of TRF treatment demonstrated that the above ratios have been substantially improved indicating the normalization of lipid parameters. Several epidemiological studies have demonstrated that elevated triglycerides is associated with increased risk of CHD (Albrink and Man, 1959; Hulley et al., 1980; Freedman et al., 1988). Our results demonstrate a significant decline in plasma triglyceride levels after 100 weeks of TRF treatment in a FH patient. In general, the hypolipidemic effect of TRF in FH patient is consistent with earlier reports, where RBO, TRF and γ-tocotrienol administration to patient with hypercholesterolemia for 4 weeks was associated with the normalization of serum and lipoprotein lipids (Raghuram *et al.*, 1989; Tan *et al.*, 1991; Qureshi *et al.*, 1995; Khan *et al.*, 1994; Beg *et al.*, 1995 & 1996). However, TRF treatment of FH patient upto 100 weeks was not associated with the normalization of plasma and lipoprotein lipid parameters, which is due to inherited genetic defect. Consistent with the reduction in triglycerides, total cholesterol and LDL-C and an increase in HDL-C concentrations, after TRF treatment, the rapid growth of skin xanthomas was arrested. In addition, regression of xanthomas on buttocks, extending to thighs, elbows and knee was observed after 20 weeks of TRF treatment.

Although treatment of FH patient with TRF is associated with a significant improvement in lipid profile including inhibition in the growth of skin xanthomas

with some regression, such patients generally require additional therapy to prevent or even reverse the damage resulting from tissue deposition caused by profoundly elevated LDL/HDL ratio. Diet plus a combination of niacin, an inhibitor of HMG-CoA reductase, and bile acid sequestrant can reduce the total and LDL concentrations in hetrozygous FH by 35-50% (Witztum *et al.*, 1989; Illingworth, 1990). Based on these findings, TRF in combination with cholestyramine will be more effective therapy for the FH patient described here.

Consistent with the hypolipidemic properties of RBO (Sharma and Rukmini, 1986; Purushothama *et al.*, 1995) or TRF in normolipidemic and hyperlipidemic rats (present study, Sharma and Rukmini, 1987; Seetharamaiah and Chandrasekhara, 1989; Watkins *et al.*, 1993) and hyperlipidemic humans (Beg *et al.*, 1997; Minhajuddin *et al.*, 1999), daily intake of 0.2 g/kg/day RBO, (equivalent to 8 mg TRF/kg/day), upto 4 weeks by normolipidemic subjects was associated with a significant decline in plasma total cholesterol and LDL-C. These results are also in agreement with previously published report indicating a hypocholesterolemic effect of palm oil concentrate containing tocotrienols, in normal and hyperlipidemic human volunteers (Tan *et al.*, 1991). Since high levels of HDL are known to be anti-atherogenic, intake of RBO (0.2 g/kg/day) by normal humans for 4 weeks caused a significant increase in HDL-C. These results are inconsistent with an earlier report (Tan *et al.*, 1991), where intake of palm oil concentrate by normolipidemic and hyperlipidemic humans for 30 days was not associated with any increase in HDL-C level. Since it has been established that LDL-C/HDL-C and HDL-C/TC (%) ratios are good predictors for the presence and severity of CAD, intake of RBO by normolipidemic subjects for 4 weeks resulted in a significant improvement in these ratios.

In the Lipid Research Clinical (LRC) Trials, every 1% decrease in serum cholesterol resulted in a 2% reduction in the risk of CHD (Lipid Research Clinic

Program, 1984 a&b). Therefore, the decline of 20% in plasma total cholesterol of familial hypercholesterolemic (FH) patient treated with TRF for 8 weeks would indicate a 40% reduction in cardiovascular risk. Similarly, a 10% decrease in LDL-C was associated with 17% reduction in CHD incidence. Based on a reduction of 24% in LDL-C after 8 weeks of TRF treatment will reflect a 41% reduction in CHD incidence. If the results of LRC Trials can be extrapolated to the present studies, dealing with hypolipidemic action of TRF in rats, a 25% decrease in plasma total cholesterol levels observed with TRF fed to hyperlipidemic rats for 1-3 weeks would indicate a 50% reduction in cardiovascular risks. Increased triglycerides level are associated with increase risk of CHD (Aberg *et al.*, 1985). In our study with FH patient, TRF treatment for 8 weeks significantly reduced the triglycerides level by 20%, thereby reducing the risk of CHD.

Based on our combined results, and earlier reported findings in normolipidemic and hyperlipidemic animal models, the TRF mediated mechanism of hypolipidemic activity in subjects with hyperlipidemia or genetic hypercholesterolemia is shown in Fig. 4.1. Administration of TRF to hyperlipidemic subjects will inhibit HMG-CoA reductase which inturn will reduce the synthesis of cholesterol. Reduced formation of cholesterol will decrease VLDL production, thereby reducing the conversion of VLDL to LDL, which will reduce total cholesterol, LDL-C and triglycerides concentrations in plasma. Based on a report (Parker *et al.*, 1993) indicating an increase in LDL-receptor protein in HepG2 cells following incubation of γ-T_3, an additional mechanism for the decreased total cholesterol, LDL-C and triglycerides levels may envolve upregulation of LDL receptors by TRF which inturn would increase the removal and catabolism of LDL. In addition, in hyperlipidemia, TRF treatment triggers a substantial increase in HDL, which would enhance the removal of excess tissue cholesterol via liver involving a HDL mediated *reverse cholesterol transport system* (Fig. 1.4).

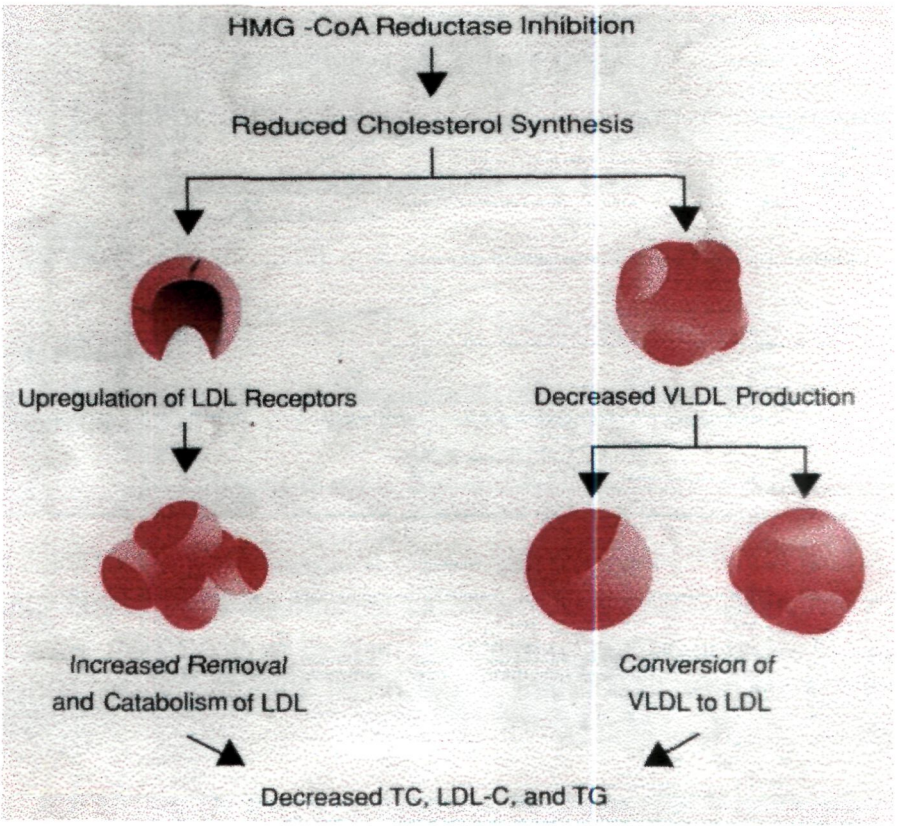

Fig. 4.1 Schematic representation for the mechanisms of hypolipidemic action of tocotrienols (TRF) mediated by inhibition of HMG-CoA reductase and/or upregulation of LDL receptors.

In summary, the combined results presented in the thesis provide sufficient evidence that administration of TRF, isolated from RBO, to normolipidemic and hyperlipidemic rats was associated with a significant decline in plasma and lipoprotein lipids including apoB. The hypolipidemic action of TRF is apparently mediated by a decrease in the enzymatic activity of HMG-CoA reductase, the rate-limiting enzyme, which in turn is modulated by a reduction in its protein mass. Formation of lipid peroxides (TBARS) and conjugated dienes (LDL oxidation) were significantly reduced in TRF treated normolipidemic rats. In response to oxidative stress, evoked in experimental hyperlipidemia in rats, as reflected by increased formation of TBARS and conjugated dienes, was significantly prevented by TRF treatment. The hypolipidemic property of TRF is dependent on the percent content of δ- and γ-tocotrienols present in TRF, which vary from cultivar to cultivar.

Long term (upto 100 weeks) TRF treatment of a FH patient with severe skin xanthomas was associated with a significant improvement in the ratios of LDL-C/HDL-C, apoB/apoA-1 and HDL-C/TC. In addition, the growth of skin xanthomas were significantly inhibited by TRF treatment. Based on the present study and previous studies in normolipidemic and hyperlipidemic humans from our laboratory, RBO or TRF (vitamin E), potent antioxidants, can be used in the prevention and treatment of hyperlipidemia and atherosclerosis. Daily intake of 8 mg TRF/kg body weight will provide maximum therapeutic benefits. In addition, daily consumption of only 0.2 g or ~0.2 ml RBO/kg/day, which is equivalent to an optimal dose of 8 mg TRF/kg/day, will prevent the occurrence of hyperlipidemia and cardiovascular diseases in normal subjects, whereas, it will be useful in the treatment of hyperlipidemia and CHD in subjects with lipid disorders.

Rice bran, an inexpensive milling by-product largely used as animal feedstock, is potentially an underutilized resource of value-added products. India is the major rice producing country in the world, but only a fraction of rice bran, rich in oil and several beneficial micronutrients, including tocotrienols and tocopherols (vitamin E), is utilized as health food. Only 0.5 million ton of RBO, corresponding to 8.5% of total vegetable oils, is consumed annually in India (*Source*: Central Organisation for Oil Industry and Trade, India, 1998). Based on TRF mediated dual therapeutic benefits, refined edible grade RBO, which has a fatty acid and glyceride composition similar to peanut oil, can be used as a major source of edible oil on daily basis. Daily use of RBO by normal population will prevent the occurrence of hyperlipidemia and cardiovascular diseases. Furthermore, daily consumption of RBO as cooking oil by subjects suffering from lipid abnormalities will be useful in the treatment of hyperlipidemia and CHD. In addition, based on avarage consumption/day/person, variety of other traditional edible oils such as mustard oil, peanut oil, soybean oil, corn oil, safflower oil, sunflower oil, etc., which lack in tocotrienols, can be supplemented with an optimal dose of 8 mg TRF/kg/day for maximal therapeutic benefits. Use of TRF-supplemented vegetable oil on daily basis will provide all the health benefits without compromising ones oil of choice. Furthermore, use of RBO, TRF or TRF-supplemented other popular edible oils will be an excellent source of vitamin E with substantial antioxidant activity.

Summary

India is the major rice producing country in the world, but only a fraction of rice bran, an inexpensive milling by-product, rich in oil and several beneficial micronutrients, including tocotrienols and tocopherols (vitamin E), is utilized as health food. Current worldwide interest in the use of tocotrienols (T_3) as potential therapeutic agent, creates a need to develop a simple and cost-effective methodology for the isolation of tocotrienol/tocopherol rich fraction (TRF) from rice bran/rice bran oil (RBO). We have developed a rapid, efficient, reproducible and cost-effective methodology for the isolation of TRF from rice bran, crude RBO or refined edible grade RBO. The TRF, thus obtained, is purified involving a single step purification procedure. The results further demonstrate that the isolation of TRF or purified TRF from crude RBO isolated from brans of two cultivars of rice, Basmati and Saket-4, was associated with a significantly higher yield of value-added products, tocotrienols and tocopherols, in comparison to TRF or purified TRF isolated from refined edible grade RBO.

Feeding of tocotrienols as TRF or purified TRF, isolated from refined edible grade RBO, to normal rats for two weeks was associated with a significant decline in plasma triglycerides, total cholesterol, LDL-C including apoB levels. HDL_2-C, which is considered as strong predictor of the presence and extent of CAD was significantly increased in TRF treated normolipidemic rats. The efficacy of TRF in the prevention of experimental hyperlipidemia in rats has been shown by administration of TRF along with atherogenic diet for three weeks, TRF significantly prevented the increase in plasma triglycerides, total cholesterol, LDL-C, apoB, HDL-C, apoA-1 and HDL_3-C levels in comparison to rats fed atherogenic diet alone. Five and seven days after the withdrawal of atherogenic diet all the plasma and lipoprotein lipid levels including apoB and apoA-1 were reduced. Treatment of hyperlipidemic rats with purified TRF resulted in a further significant reduction in the above parameters indicating the efficacy of TRF in the

treatment of experimental hyperlipidemia. The minimum dose of TRF or purified TRF required to exert the maximum hypolipidemic effect in normolipidemic and hyperlipidemic rats has been found to be 8 mg TRF or 5.2 mg purified TRF/kg/day.

. The results provide evidence that administration of TRF or purified TRF to normolipidemic rats was associated with a significant decrease in enzymatic activity of HMG-CoA reductase. Experimental hyperlipidemia in rats was associated with down-regulation of enzyme activity and protein mass of HMG-CoA reductase. Administration of purified TRF along with atherogenic diet to rats was associated with further inhibition of enzymatic activity of HMG-CoA reductase and its protein mass, in comparison to hyperlipidemic controls, indicating the possibility of a different mechanism of HMG-CoA reductase inhibition by TRF. Five and seven days of purified TRF treatment to hyperlipidemic rats, after the withdrawal of atherogenic diet, caused a further reduction in the activity and protein mass of HMG-CoA reductase, in comparison to untreated hyperlipidemic rats. A concomitant decline in immuno-reactive HMG-CoA reductase protein mass represents an initial demonstration and indicates the involvement of long-term control by alteration in protein synthesis and/or degradation. Based on the combined results, it can be concluded that cholesterol lowering property of TRF in normolipidemic and hyperlipidemic rats is due to the suppression of enzymatic activity and protein mass of HMG-CoA reductase.

Administration of TRF, enriched with tocotrienols and tocopherols, resulted in a significant decline in microsomal lipid peroxidation (TBARS) and plasma LDL oxidation (conjugated dienes) in normolipidemic and hyperlipidemic rats. In response to oxidative stress, evoked in experimental hyperlipidemia in rats, as reflected by increased formation of TBARS and conjugated dienes, was substantially blocked by TRF feeding along with atherogenic diet, to a level lower

than the observed values of normolipidemic rats. Treatment of hyperlipidemic rats with purified TRF for 5 and 7 days not only caused significant decline in lipid peroxides and conjugated dienes in comparison to hyperlipidemic untreated control rats, but further reduced these levels below normal values. These results underline the importance of TRF as a potent antioxidant agent. The combined results demonstrate that strong hypolipidemic impacts of TRF in conjunction with its potent antioxidant property can provide an additional therapeutic benefit in the prevention and treatment of hyperlipidemia and coronary heart disease.

The results also demonstrate a differential hypolipidemic impact of purified TRF isolated from two cultivars of rice, raw Basmati and Saket-4, due to the difference in their γ- and δ-T_3 content in hyperlipidemic rats. Based on the total content of γ- and δ-T_3 present in the purified TRF of each cultivar, hypolipidemic efficiencies at a dose of 3 and 6 mg equivalent TRF/kg/day (calculated on the basis of combined content γ- and δ-T_3 present in 3 and 6 mg TRF from refined edible grade RBO), caused a dose-dependent decline in plasma and lipoprotein lipids including apoB as well as enzymatic activity and protein mass of HMG-CoA reductase, in hyperlipidemic rats after 7 days of treatment. Thus, efficacy of hypolipidemic action of TRF or purified TRF is directly related to the total content of γ- and δ-tocotrienol present in TRF or purified TRF isolated from crude RBO of different cultivars of rice or from refined edible grade RBO. Consistent with earlier results, administration of 3 and 6 mg equivalent doses of purified TRF/kg/ day from two cultivars of rice to hyperlipidemic rats for 7 days significantly reduced the formation of TBARS and conjugated dienes in a dose-dependent manner.

Drugs targeted to inhibit HMG-CoA reductase activity are extensively used to control hyperlipidemia including familial hypercholesterolemia (FH). Therefore,

based on hypolipidemic property of TRF a long-term therapy of a FH patient with severe skin xanthomas was undertaken. TRF treatment at a dose of 8 mg/kg/day caused a significant reduction in triglycerides, total cholesterol, LDL-C and apoB levels. There was a substantial increase in the levels of HDL-C (155%) and apoA-1 (166%). A highly significant increase in HDL subfraction, HDL_2-C (620%) was seen after 100 weeks of TRF treatment. TRF also caused a substantial improvement in the ratios of LDL-C/HDL-C, apoB/apoA-1 and HDL-C/total cholesterol, indicating the normalization of lipid parameters. Consistent with the reduction in triglycerides, total cholesterol and LDL-C concentrations and an increase in antiatherogenic HDL level, after TRF treatment, the rapid growth of skin xanthomas was arrested. In addition, a significant regression of xanthomas on buttocks extending to thighs, elbows and knee was observed after 20 weeks of TRF treatment.

Similar to hypolipidemic impact of TRF isolated from RBO, in normolipidemic rats, intake of RBO by normolipidemic humans for 4 weeks was associated with a significant decline in plasma total cholesterol and LDL-C levels, with a significant increase in HDL-C level. Intake of RBO was also associated with a significant improvement in already normal ratios of LDL-C/HDL-C and HDL-C/TC, indicating a strong antiatherogenic property of RBO, rich in tocotrienols.

Our combined results demonstrate a strong hypolipidemic and antioxidant action of TRF and purified TRF isolated from refined edible grade RBO or crude RBO, when administered to normolipidemic and hyperlipidemic rats. Similar to hypolipidemic effects of TRF in the rats, total cholesterol, LDL-C and apoB, which are positively associated with coronary heart disease, were significantly reduced in FH patient treated with TRF. Levels of plasma HDL-C and apoA-1, which are considered as anti-atherogenic, were significantly increased. In addition,

which are positively associated with coronary heart disease, were significantly reduced in FH patient treated with TRF. Levels of plasma HDL-C and apoA-1, which are considered as anti-atherogenic, were significantly increased. In addition, HDL_2-C, which is considered as strong predictor of the presence and extent of CAD was significantly increased.

Based on TRF-mediated dual therapeutic benefits, refined edible grade RBO, which has a fatty acid and glyceride composition similar to peanut oil, can be used as a major source of edible oil on daily basis. Daily use of RBO by normal population will prevent the occurrence of hyperlipidemia and cardiovascular diseases. Furthermore, daily consumption of RBO as cooking oil by subjects suffering from lipid abnormalities will be useful in the treatment of hyperlipidemia and CHD. In addition, variety of other traditional edible oils such as mustard oil, peanut oil, soybean oil, corn oil, safflower oil, sunflower oil, etc., which lack in tocotrienols, can be supplemented with an optimal dose of 8 mg TRF/kg/day for maximal therapeutic benefits. Use of TRF-supplemented vegetable oil on daily basis will provide all the health benefits without compromising ones oil of choice. Furthermore, use of RBO, TRF or TRF-supplemented other edible oils will be an excellent source of vitamin E with substantial antioxidant activity.

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